



PHD

A study of protein antigenicity using monoclonal antibodies against citrate synthase

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(i)

A STUDY OF PROTEIN ANTIGENICITY
USING MONOCLONAL ANTIBODIES
AGAINST CITRATE SYNTHASE

Submitted by
DAVID MARK BRENNAND
for the degree of PhD
of the
University of Bath
1987

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(iii)

ABSTRACT

This thesis describes a study of the antigenicity of pig heart citrate synthase (PHCS).

Following immunisation with PHCS, 11 mouse monoclonal antibodies (mAbs) were raised against this enzyme, two of IgG1 type, and nine of IgM type.

An investigation was performed into the effectiveness of a variety of solid-phase immunoassay systems in the detection of mAbs. This work showed that a sandwich ELISA was the most effective assay for detecting all of a library of mAbs raised against PHCS.

Epitope mapping studies performed on fragments of PHCS (produced by enzymic and chemical cleavage) have shown that mAbs B and C recognise an epitope between residues 258-313, and mAb E recognises an epitope between residues 1-230. Further studies using a synthetic peptide have shown that the epitopes recognised by mAbs B and C lie between residues 288-302.

Methods to predict potential antigenic regions including use of hydrophilicity data, atomic mobility values, composite surface profile and protrusion indices were applied to PHCS. All four methods indicated that a possible antigenic site should exist at the experimentally determined epitope between residues 288-302 of PHCS. This correlation confirms the ability of these predictive methods to indicate possible antigenic sites.

(iv)

TO

MY MOTHER AND FATHER

ABBREVIATIONS

A ₄₀₅	-Absorbance at 405 nm
AChR	-Acetylcholine receptor
ADP	-Adenosine diphosphate
ATP	-Adenosine triphosphate
APC	-Antigen presenting cell
Ala	-Alanine
Arg	-Arginine
Asn	-Asparagine
Asp	-Aspartic acid
B cell	-Bursa derived lymphocyte
BME	-Basal medium, Eagles
<u>B.megaterium</u>	<u>-Bacillus megaterium</u>
B.value	-X-ray crystallographic temperature factor
CS	-Citrate synthase
CoA	-Coenzyme A
Cys	-Cysteine
Cyt	-Cytochrome
DMSO	-Dimethylsulphoxide
DNA	-Deoxyribonucleic acid
DNP	-Dinitrophenol
DTNB	-5,5'-dithiobis(2-nitrobenzoic acid)
<u>E.coli</u>	<u>-Escherichia coli</u>
EDTA	-Ethylenediaminetetraacetic acid
ELISA	-Enzyme linked immunosorbant assay
FAD	-Flavin adenine dinucleotide

FCA	-Freund's complete adjuvant
FCS	-Foetal calf serum
GDP	-Guanosine diphosphate
Gly	-Glycine
GTP	-Guanosine triphosphate
HAT	-Hypoxanthine, aminopterin and thymidine
Hb	-Haemoglobin
His	-Histidine
HLA	-Human leukocyte antigen
HPLC	-High performance liquid chromatography
HT	-Hypoxanthine and thymidine
Ile	-Isoleucine
Influenza HA	-Influenza haemagglutinin
Leu	-Leucine
Lys	-Lysine
mAb	-Monoclonal antibody
Mb	-Myoglobin
MOPS	-3-(N-Morpholino)propanesulphonic acid
mRNA	-Messenger ribonucleic acid
NAD	-Nicotinamide-adenine dinucleotide
NADP	-Nicotinamide-adenine dinucleotide phosphate
PAGE	-Polyacrylamide gel electrophoresis
PBS	-Phosphate buffered saline
PEG	-Polyethylene glycol

PHCS	-Pig heart citrate synthase
Phe	-Phenylalanine
PI	-Protrusion index
Pro	-Proline
rRNA	-Ribosomal ribonucleic acid
SDS	-Sodium dodecyl sulphate
Ser	-Serine
SwMb	-Sperm whale myoglobin
T cell	-Thymus derived lymphocyte
TEMED	-N,N,N',N'-tetramethylethylene diamine
T _H cell	-T helper cell
Thr	-Threonine
TMVP	-Tobacco mosaic virus protein
Trp	-Tryptophan
T _S cell	-T supressor cell
Val	-Valine
YCS	-Yeast citrate synthase

TABLE OF CONTENTS

	page
Title	(i)
Acknowledgements	(ii)
Abstract	(iii)
Dedication	(iv)
Abbreviations	(v)
Table of contents	(viii)
<u>CHAPTER 1</u> <u>GENERAL CONTENTS</u>	1
1.1 The Immune Response	4
1.2 Protein Antigenic Structure	6
1.3 Monoclonal Antibodies	15
1.4 Strategies for Predicting Protein Antigenic Sites	23
<u>CHAPTER 2</u> <u>CITRATE SYNTHASE</u>	33
2.1 The Citric Acid Cycle	34
2.2 Citrate Synthase	36
2.3 Species Dependent Properties of Citrate Synthase	38
2.4 Primary Sequence Data for Citrate Synthase from Pig Heart, <u>E.coli</u> , and Yeast	42
2.5 Physical Structure of Citrate Synthase	45
2.6 Immunochemical Study of Citrate Synthase	48
Aims of This Study	50

	page
<u>CHAPTER 3</u> <u>MATERIALS AND METHODS</u>	51
Materials	54
Methods	56
 <u>CHAPTER 4</u> <u>PRODUCTION OF MONOCLONAL ANTIBODIES</u>	 84
4.1 Comparison of immunisation procedures	85
4.2 Production of mAb secreting cell lines	
A-E	85
4.3 Production of mAb secreting cell lines	
F,G,H,K,L,and M	87
4.4 Production of mAbs from ascitic fluid	89
4.5 MAb sub-class typing	89
4.6 Further purification of mAbs	92
4.7 Purity of mAbs	93
4.8 Discussion	95
 <u>CHAPTER 5</u> <u>IMMUNOCHEMICAL SCREENING METHODS</u>	 100
5.1 Choice of optimum conditions for ELISAs	
and Immunodot blots	101
5.2 Hybrid screening using ELISAs IV and VI	104
5.3 Comparison of solid phase immunoassays	104
5.4 'Open' and 'Closed' forms of PHCS used	
in sandwich ELISAs	123
5.5 The effect of SDS and 2-mercaptoethanol	
on the presentation of PHCS in	
sandwich ELISAs	127
5.6 Discussion	130

	page
<u>CHAPTER 6</u> <u>PEPTIDE MAPPING OF MABS</u>	134
6.1 Limited proteolysis of PHCS using chymotrypsin, trypsin, and subtilisin	135
6.2 Peptide mapping of mAbs to proteolytic fragments of PHCS	137
6.3 Further peptide mapping of mAbs to a tryptic digest of PHCS	142
6.4 Cleavage of asp-pro bonds by dilute acid	147
6.5 Peptide mapping of mAbs to acid cleaved fragments of PHCS	147
6.6 MAb immunosorbent chromatography performed on PHCS fragmented at asp-pro bonds	154
6.7 Prediction of the products formed by cleavage of PHCS at asp-pro bonds	158
6.8 Peptide mapping of mAbs to PHCS fragmented at asn-gly bonds	160
6.9 Discussion	162
 <u>CHAPTER 7</u> <u>COMPUTER PREDICTIONS OF THE</u> <u>EPITOPES PRESENT ON PHCS</u>	 171
7.1 Introduction	172
7.2 Prediction of the epitopes of PHCS using hydrophilicity data	172
7.3 Prediction of the epitopes of PHCS using 'Composite surface profile' data	175

	page
7.4 Prediction of the epitopes of PHCS using atomic mobility values	177
7.5 Prediction of the epitopes of PHCS using 'Protrusion indices'	178
7.7 Discussion	183
<u>CHAPTER 8</u> <u>CONCLUDING REMARKS</u>	186
<u>CHAPTER 9</u> <u>APPENDIX</u>	192

<u>CHAPTER ONE</u>	<u>INTRODUCTION</u>	Page
1.0	General Introduction	2
1.1	The Immune Response	4
1.2	Protein Antigenic Structure	6
1.2.1	Determination of the antigenic structure of sperm whale myoglobin	7
1.2.2	Determination of the antigenic structure of hen egg lysozyme	9
1.2.3	The effect of substitutions, and the role of conformation on antigenicity	11
1.2.4	Level of antibody responses to regions outside the main antigenic sites	13
1.2.5	Summary	13
1.3	Monoclonal Antibodies	15
1.3.1	Epitope analysis using monoclonal antibodies	16
1.3.2	The study of acetylcholine receptor using monoclonal antibodies	19
1.3.3	Hexokinase A	21
1.3.4	Summary	22
1.4	Strategies for Predicting Protein Antigenic Sites	23
1.4.1	Accessibility	24
1.4.2	Protrusion Index	25
1.4.3	Hydrophilicity	26
1.4.4	Mobility	27
1.4.5	Composite Surface Profile	30
1.4.6	Structurally inherent antigenic sites	31

1.0 General Introduction

A current major area of research in immunology is the study of the antigenicity of proteins. At present the directions of this study are towards:

a) experimentally determining the structures and amino acid compositions of individual antigenic determinants

b) devising computer programmes to predict which segments of protein molecules will be involved in antigenic determinants

c) using chemically synthesised immunogens to initiate the production of antibodies or activated T cells which can react with larger antigens.

A detailed study of the specificities and affinities of antibodies for antigenic determinants will help us to choose the best antibodies for use as tools in sensitive immunoassays, affinity purification procedures and to evaluate their use for in vivo drug targetting. The ability to predict and chemically synthesise antigenic sites for use as immunogens may lead to their general use in vaccine production.

Only a limited number of proteins have so far been studied by these methods.

This project is concerned with the study of the antigenic determinants of citrate synthase, using monoclonal antibodies and experimental analyses of antigenic sites. Results are compared with computer predictions of the antigenic determinants for the enzyme.

Citrate synthase is a well characterized protein and provides an ideal subject by which to increase the overall database of information on protein antigenic structures.

1.1 The Immune Response

The site on an antigen to which an antibody binds is called the antigenic determinant (also called an epitope, or antigenic site).

Immunogenicity is a term used to describe the ability of an antigen to elicit a response, following immunisation, generating antibodies and activated T cells. The antigenicity of a protein is its ability to be recognised by the product of a previous response, by antibodies or T cells. Any antigen which is immunogenic must be antigenic. However, some low molecular weight substances, such as drugs, hormones and other small molecules are unable to stimulate a primary response and are thus non-immunogenic unless conjugated to larger 'carrier' molecules; such substances are termed 'haptens'. The haptens alone, however, are able to react with the resultant antiserum, or sensitized cells, and are therefore antigenic.

An immune response leading to the production of antibodies has two phases;

- 1) the recognition of the antigen as foreign, a function which is usually fulfilled by T lymphocytes and macrophages.
- 2) the effector phase in which antibodies are produced and the antigen eliminated.

Very few antigens bind directly to antigen-reactive T cells or B cells, rather they are usually presented on the surface of antigen presenting cells (APCs). APCs include dendritic cells, which are non-

phagocytic, and macrophages, which are phagocytic. Thus the antigen may be presented to T and B cells as a native, or partially denatured antigen.

Each B cell is committed to the production of antibodies with a unique combining site which binds to one epitope only. During antigen presentation the B cell recognises the antigen through its receptor and proliferates into both memory cells and antibody-secreting plasma cells. This proliferation of one cell leading to the production of a population of cells is called clonal expansion. T cells do not secrete antibodies, but as do B cells, they have surface receptors to specific antigenic epitopes and are similarly expanded clonally. Helper T cells which have responded to antigen presented by APCs subsequently activate B cells, already committed to the same antigen, into antibody production. This effect is balanced by suppressor T cells, which appear to be able to bind specific antigens directly, without the need for APCs, and subsequently inhibit T-helper cells, rather than acting directly on B cells. These regulatory T cells appear to exert this control of the production of antibody by B cells through the release of soluble mediating factors.

1.2 Protein Antigenic Structure

This section describes the discovery of different types of antigenic determinants, using experiments employing serum antibodies.

Early workers used peptide fragments to study protein immunochemistry. As early as 1942, Landsteiner showed how peptides from silk fibroin were able to inhibit the reaction of the latter with its antibodies.

Work by the group of Atassi from 1963-1975 led to the elucidation of the first complete antigenic structure of a protein, that of sperm whale myoglobin (swMb). This same group also determined the antigenic sites of hen egg lysozyme (Atassi, 1978), serum albumen (Atassi, 1982) and human adult haemoglobin (Hb) (Atassi, 1984). Due to the complexity of protein structure, the antigenic sites cannot be determined by the exclusive application of a single approach. Atassi, 1975, developed a strategy consisting of five approaches. These are:

- 1) The determination of the influence of conformational changes on the immunochemical behaviour of the protein

- 2) The study of the immunochemistry of specific chemically modified derivatives of the protein

- 3) The isolation and the study of a large number of overlapping peptide fragments to identify immunochemically-reactive fragments that can quantitatively account for the total anti-protein antibodies

- 4) The study of the immunochemistry and conformation of specific chemical derivatives of

immunochemically reactive peptides.

5) The synthesis, chemically, of the reactive regions defined by methods 1-4, and verification of the antigenic activity of these peptides.

Thus, determination of the antigenic structures of swMb and lysozyme has defined the problems involved in precise delineation of two types of antigen sites for subsequent workers studying protein antigenicity.

1.2.1 Determination of the antigenic structure of sperm whale myoglobin

Sperm whale myoglobin is a protein of 153 amino acids folded in a helical compact structure, carrying 1 haem group.

Conformational changes in swMb caused by inclusion of modified haem groups, or modification of side chains were shown to influence antigenic activity (Andres and Atassi, 1970). A series of well purified derivatives modified at a total of 23 amino acid sites gave an initial indication of the location of some of the amino acids needed for antibody binding. However, care was needed to ensure that a resultant decrease in antibody binding following modification was directly due to alteration of an essential antigenic site residue and not caused indirectly by a conformational change (Atassi and Thomas, 1969; Atassi, 1975).

Peptide fragments of swMb and derivatives of

the same were tested for their ability to react with antisera raised against the native swMb (Singhal and Atassi, 1971; Atassi, 1975). Following this initial description of the epitopes, synthetic peptides corresponding to these regions were tested for their ability to inhibit immunoprecipitation of the native swMb by antisera (Pai and Atassi, 1975; Kokotsu and Atassi, 1974).

By this method native swMb was shown to have 5 major antigenic sites, each consisting of a linear amino acid sequence, seen to be sequentially linked by peptide bonds. This type of antigenic site is termed 'continuous' (also termed a linear epitope). Two of the sites exhibit degrees of shift or displacement and minor variability in size (limited to ± 1 residue only) from one antiserum to the next. The sites contain 6-7 residues and are between 1.9-2.3nm in their extended dimensions (Atassi, 1984). The size, surface locations and shape of these antigenic sites make them quite accessible for binding with antibody combining sites. Due to their content of hydrophilic residues (lysine, arginine, aspartate, glutamate and histidine), it was concluded that the interaction with antibodies must be predominantly polar in nature. Other stabilizing effects are contributed by hydroxy and non-polar amino acids through hydrogen bonding and hydrophobic interactions (Atassi, 1984).

The same five antigenic sites of sw myoglobin are recognised by rabbit, goat, chicken, cat, pig and mouse antisera (Twining et al, 1980).

1.2.2 Determination of the antigenic structure of Hen Egg Lysozyme

Hen egg lysozyme is of a different structural type when compared to Mb. It has 129 amino acid residues and is held together in a tight conformation by 4 internal disulphide crosslinks. Lysozyme has 25% of its residues forming α -helices, as compared to 70% for myoglobin.

Initial work using the previously proposed strategy proved difficult, partly due to the tight conformation making specific cleavage to fragments unreliable and partly due to the apparent need for the disulphide bonds to be intact in order to retain antigenicity (Lee and Atassi, 1973; Habeeb and Atassi, 1971a).

This initial work predicted 3 antigenic sites. Attempts were made to chemically synthesise regions corresponding to these sites and containing the disulphide bonds, but this proved too difficult for routine use (Atassi et al, 1975). Diglycyl segments, however, were found to be ideal replacements for disulphides in chemical synthesis experiments. This newly developed concept of chemically synthesising a region where the constituent amino acids are not generally found sequentially linked by peptide bonds in the native protein was called 'surface-simulation synthesis' (Atassi et al, 1976a).

For antigenic site 2, the initially implicated residues from chemical modification experiments are shown in fig 1(a) (Lee and Atassi, 1975; Atassi et al, 1976b).

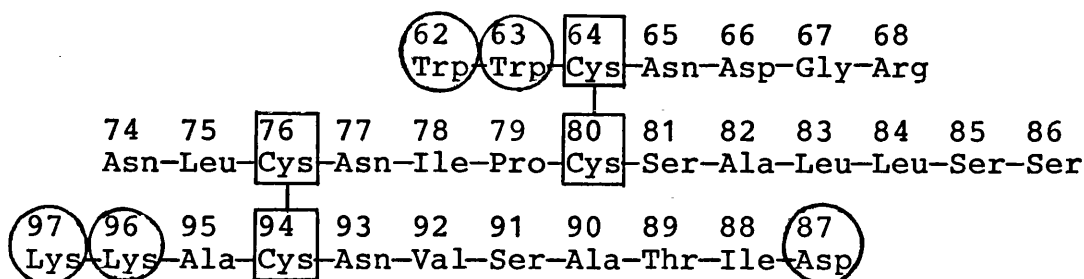
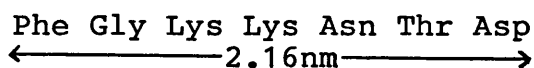
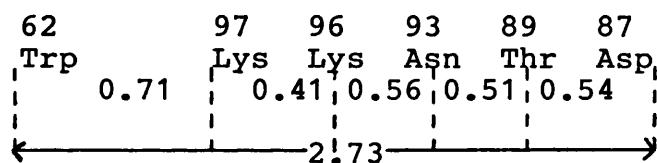
Fig.1aFig.1b Synthetic SiteFig.1c Constituent Residues

Fig.1a shows the residues (ringed) thought to be important for the antigenicity of site 2 of hen egg lysozyme following fragmentation work.

Fig.1b shows the synthetic site found to carry the full reactivity of site 2.

Fig.1c shows the constituent residues of the native site 2 of hen egg lysozyme. The distances (in nm) separating the constituent residues and the overall dimension of the site (in its extended form) are given, together with the dimension of the surface - simulation site.

Following synthesis of a variety of peptides of the type shown in fig 1(b), the final constituent residues of the antigenic site, fig 1(c), were delineated (Lee and Atassi, 1976; Atassi, 1978). This was the first antigenic determinant shown to comprise of surface residues in close proximity by virtue of the folding of the polypeptide chain, but which are not sequentially linked by peptide bonds. This type of epitope is called a discontinuous antigenic determinant (sometimes termed topographic).

All three major antigenic sites of lysozyme are found to be discontinuous, they each contain 5-6 residues and are between 2.1-3.0nm in their extended dimensions.

1.2.3 The effect of substitutions, and the role of conformation on antigenicity

Conformational changes intentionally imposed on Hb and Mb by chemical alterations in the haem moiety (outside the antigenic site) lead in each protein to alterations in its antigenic reactivity (Atassi, 1967; Reichlin et al, 1963).

Using cross-reactivity data for many well defined protein systems White et al, 1978, showed how residues subject to evolutionary substitution in proteins exert an influence on antigenicity. This may be by directly altering a reactive site, or by exerting indirect conformational changes in the

determinant (East et al, 1980).

Lysozyme and α -lactalbumin, 2 highly homologous proteins, do not exhibit any immunochemical cross-reactivity because of conformational differences between the two proteins (Habeeb and Atassi, 1971b). However, when unfolded by reduction or alkylation, the 2 proteins exhibit the expected immunochemical cross-reactivity with antisera raised against either unfolded protein (Arnon and Maron, 1971).

Lee and Atassi, 1977, showed that nitration of a tyrosine outside an antigenic site of lysozyme creates an electrostatic inductive effect which disrupts the reactivity of that site. No detectable conformational changes were associated with this modification (Atassi, 1978).

In myoglobin, 22% of residues are involved in antigenic sites, and yet 80% of substitutions are immunologically detectable (Berzofsky et al, 1982). Residues as far away as 1.6nm distant from an antigenic site have been shown to exert conformational restrictions necessary for antibody binding at the site (Twining et al, 1980). If all the residues within 0.7nm of an antigenic site were arbitrarily considered to be important environmental residues, an additional 56% of Mb residues, (giving a total of 78%) would be found to exert effects on its antigenicity (Kazim and Atassi, 1980).

Thus conformational changes in an antigen brought about by chemical modification or evolutionary

change have been shown to be of great importance in influencing antigenicity. The extent and nature of conformational changes has a variable effect on antigenicity depending on the protein (Andres and Atassi, 1970; Atassi et al, 1972).

1.2.4 Level of antibody responses to regions outside the main antigenic sites

Although epitope studies of swMb and hen egg lysozyme account for approximately 98% of the antibodies raised against these native proteins, there is a low level of antibody response (approximately 2%) versus other regions (Atassi, 1980; Atassi and Lee, 1978). Dean and Schechter, 1979, found a subpopulation of less than 1% of total anti-Hb antibodies bound to an α 129-141 epitope. This could only be detected when a large excess of antibody was used.

1.2.5 Summary

This section has discussed the discovery of the existence of two types of antigenic site, using polyclonal antibodies raised against native proteins. Myoglobin contains five major antigenic sites of the continuous type, lysozyme has three major antigenic sites of the discontinuous type.

The use of monoclonal antibodies (mAbs) discussed in the following section, enables the examination of the overall antibody response in finer detail. This includes the ability to study minor antibody responses contained in serum antibodies, see section 1.2.4.

1.3 Monoclonal Antibodies

The immune response has evolved to combine the maximum efficiency of clearing specific foreign antigens from the host, with the least likelihood of reacting with self. Dependent on the regulation of Immune response (Ir) genes and balance of T_H/T_S cell activity to each possible epitope, a polyclonal serum is produced which is a disproportionate mixture of a variety of monoclonal antibodies, against the sum of the various epitopes of the antigen. The polyclonal serum contains monoclonal antibodies of various classes and sub-classes, with various affinities to each epitope present on the antigen.

The development of hybridoma techniques, to select and immortalize individual monoclonal antibody secreting cells (Kohler and Milstein, 1975) has allowed the dissection of polyclonal serum into its individual components. Normal antibody-producing B lymphocytes do not grow readily in culture. However, when fused with a myeloma cell line (derived from a B-lymphocyte malignancy) a hybridoma is produced which secretes the antibody of the primed B cell, yet can be grown indefinitely in culture.

This permits amplification in vitro of what would ordinarily constitute trace antibody responses in vivo. By definition a monoclonal antibody preparation contains antibodies of only one type and specificity.

The potential uses of monoclonal antibodies in biological systems are immense and they have been raised to a diverse list of molecules, see table 1.

Due to their specificity, mAbs are used as biological tools and markers in purification, quantitation, structure/function studies, and histology, see table 2.

MABs may also be used for the polysome purification of specific mRNAs, for molecular cloning and for purification of translated products. In vivo, the use of mAbs tagged to cytotoxic agents offers potentially exciting selective cancer therapy, or drug targeting. An example of a target may be the enzyme xanthine oxidase for treatment of breast carcinoma (Vora, 1985).

1.3.1 Epitope analysis using monoclonal antibodies

Studies of the antigenic determinants bound by monoclonal antibodies have predominantly been restricted to those concerning continuous epitopes. The difficulty of examining discontinuous epitopes has been emphasised in section 1.2.2.

Peptide mapping studies using mAbs make wide use of the immunoblotting procedure of Towbin et al, 1979, whereby protein fragments are electrophoretically transferred from polyacrylamide gels to nitrocellulose sheets and the reactive bands probed with antibodies. Antigenetically reactive fragments can also be identified by their retention on mAb immunoabsorbent columns

Table 1 Examples of the diverse list of antigens to
which monoclonal antibodies have been raised.

<u>ANTIGEN</u>	<u>REFERENCE</u>
DNP (a hapten)	Eshhar <u>et al</u> , 1980
Human Growth Hormone	Ivanyi and Davies, 1980 Surowy <u>et al</u> , 1984
DNA	Andrzejewski <u>et al</u> , 1980
rRNA	Eilat <u>et al</u> , 1980
Muscarinic Cholinergic Receptor	Venter <u>et al</u> , 1984
HLA antigens	Brown <u>et al</u> , 1979 Yu <u>et al</u> , 1980
Substance P	Cierniewski <u>et al</u> , 1984
Influenza Virus	
Neuraminidase	Webster <u>et al</u> , 1982
Creatine Kinase	Morris <u>et al</u> , 1985
Tryptophan Synthase	Friguet <u>et al</u> , 1984

Table 2 Examples of the uses of monoclonal antibodies

<u>USE</u>	<u>ANTIGEN</u>	<u>REFERENCE</u>
Immunopurification	choline acetyltransferase	Crawford <u>et al</u> , 1982
	Ca ²⁺ -calmodulin phospho- diesterase	Hansen and Beavo 1982
Immunoassay (quantitation)	Alkaline phosphatase	Slaughter <u>et al</u> , 1981
	Prostatic acid phosphatase	Lillehoj <u>et al</u> , 1982
	Human Chorionic Somatomammotropin	Mariani <u>et al</u> , 1984
	Phosphofructokinase	Davidson <u>et al</u> , 1983
Isoenzyme studies	Cyt P-450	Sesardic <u>et al</u> , 1986
	β-D-galactosidase	Frackelton and Rotnam, 1980
Investigation of enzyme activity	Tryptophan Synthase	Djavadi-Ohanian <u>et al</u> , 1986
Antigenic mapping	Influenza Virus Neuraminidase	Webster <u>et al</u> , 1982
	Creatine Kinase	Morris <u>et al</u> , 1985
Immunocytochemistry	DNA polymerase	Berisch <u>et al</u> , 1982
Electron Microscopic localisation	Acetyl Choline Receptor	Ratnam <u>et al</u> , 1986a
Assignment to chromosome	Phosphofructokinase L K	Vora and Francke, 1981

(Duffy and Kurosky, 1982), or by their precipitation using antibody. Readily purified fragments may also be probed with antibodies in radioimmunoassays and enzyme-linked immunosorbent assays (ELISA), to test for reactivity.

Two examples of protein studies performed using monoclonal antibodies are those of the acetylcholine receptor (Ratnam et al, 1986a), and hexokinase A (Ureta et al, 1986). These will be discussed to illustrate some of the uses of mAbs in performing structure/function studies of proteins.

1.3.2 The study of acetylcholine receptor using monoclonal antibodies

The nicotinic acetylcholine receptor of Torpedo californica is composed of four types of glycoprotein subunit in an $\alpha_2\beta\gamma\delta$ stoichiometry (Raftery et al, 1980). The receptor is a cation channel in the membrane and is regulated by the neurotransmitter, acetylcholine (Neher and Sakman, 1976). Significant homology has been shown between the subunits, the amino acid sequences of which are known. Structurally all the subunit C-termini have been shown to be intracellular (Young et al, 1985).

Over 200 anti-AChR mAbs have been raised (Criado et al, 1985; Mochly-Rosen and Fuchs, 1981). A few mAbs have been shown to inhibit receptor function noncompetitively (Blatt et al, 1986).

Monoclonal antibodies have become available which bind specifically to the C-terminal decapeptides of subunits α , β and δ ; the γ subunit has not yet been studied (Lindstrom et al, 1984). Fragments of the subunits have been produced by digestion with staphylococcal V8 protease, and papain, separated on a discontinuous polyacrylamide gel electrophoretic system and electroblotted onto diaminophenyl thioether paper (Ratnam et al, 1986a; Souroujon, 1986). Blots have been probed with monoclonal antibodies against the carboxy-terminal decapeptides of the α , β and δ subunits. This identified the carboxy terminal fragments, and by assuming a mean residue weight of 115, fragments were assigned to appropriate amino acid sequences. After probing the blots with anti-AChR mAbs, it was possible to map the binding of these mAbs onto the amino acid sequences of the AChR subunits. In this way, the main immunogenic region of the native receptor which is of pathological importance in the autoimmune disease myasthenia gravis has been mapped (Ratnam et al, 1986a).

Further studies of the AChR using mAbs are in three main areas:

a) Using colloidal gold-protein A as a means of visualizing mAbs bound to vesicles under electron microscopy, information has been obtained on the transmembrane orientation of the polypeptide chains of the receptor within the membrane (Ratnam et al, 1986b).

b) Regions of the primary sequence of AChR with a direct role in its physiological functioning can be identified by mapping mAbs that affect the channel activity. However, these studies are difficult to interpret (Wari and Lindstrom, 1985; Blatt et al, 1986).

c) Mapping the overall antigenic structure of the receptor will enable the pinpointing of peptide sequences that play critical pathogenic roles in the autoimmune disease myasthenia gravis (Lindstrom, 1985).

This work shows how mAbs are used to elucidate antigenic epitopes, study the orientation and structure of complex proteins and examine structure/function relationships.

1.3.3 Hexokinase A

A library of seven mouse mAbs was raised against rat brain hexokinase (Finney et al, 1984). Using a direct coating ELISA, 3 mAbs were shown to react with rat, mouse, guinea pig, cat, dog, sheep, pig, cow, rabbit and chicken brain hexokinase, showing wide species conservation at these epitopes. The other epitopes differed markedly in species distribution, although the direct ELISA may not be the best assay to use for cross-reactivity studies.

Peptide mapping (after limited tryptic digestion) in conjunction with immunoblotting techniques employing mAbs, showed the domain structures of the

various species' hexokinases to be similar to those of rat hexokinase. However, the domain sizes varied between the species (Ureta et al, 1986; Wilson and Smith, 1985). None of the mAbs affects the enzyme activity, which suggested that they bound at sites spatially distinct from the active site (Finney et al, 1984). This work shows how mAbs can be used to study enzymic structure/function relationships and to examine epitope diversity across species.

1.3.4 Summary

A mAb preparation contains only one antibody, representing an individual component of a polyclonal serum. A polyclonal antiserum will also contain antibodies versus other antigens as well as those against the antigen used to immunise the host.

Use of mAbs enables the investigation of the individual epitopes present on an antigen with a specific probe. Hence, the individual epitopes can be examined for their species conservation, or their importance in the function of the protein (i.e. enzyme activity). Due to their specificity, mAbs are far superior to antisera for use as immunochemical markers, for affinity purification and in immunoassays.

1.4 Strategies for Predicting Protein Antigenic Sites

The question of what an antibody actually 'sees' when it recognises its antigen has not yet been fully answered. The total surface available on the antibody for antigen contact is large, about 4 x 5nm (Rees and de la Paz, 1986). Precisely how much of this surface is utilized obviously depends upon the size and shape of the antigen. Some antibodies have been shown to be capable of binding to single amino acids and dipeptides (Geysen, 1985), although the optimal epitope size to maximise antibody binding efficiency is generally accepted as being between 5-10 residues (Amit et al, 1985).

The analysis of which parameters are involved in antigen-antibody interactions and the selection of optimal sequences to use as synthetic immunogens is only readily performed for continuous epitopes, leaving discontinuous sites, as yet, largely uncharted.

Experimental analysis of antigenic sites is an arduous procedure. Thus, much work is presently being performed to try and find a predictive approach for locating the sites on a protein which are antigenically important. Following correlation studies of known protein antigenic structures (found by experimental means) with various physical parameters, using computer analysis, some predictive methods have been suggested. The parameters used include accessibility,

surface protrusion, hydrophilicity, mobility and structurally inherent antigenicity. They are discussed in the following sections.

1.4.1 Accessibility

For antibodies to bind a protein antigen in solution, antigenic sites should be accessible on the surface of the native protein, consistent with the work of Atassi, 1984. Molecular surface calculations can be performed on a protein of known structure by mathematically rolling a solvent water molecule over the van der Waal's surface of the protein (Hubbard and Ivatt, 1981; Rice et al, 1982).

Exceptions to this requirement for antigenicity however have been found. When a mAb isolated from a mouse immunized with native swMb was used in a competitive ELISA, native swMb failed to compete for antibody with swMb bound directly to an ELISA plate. The mAb did however bind swMb, horse Mb and a synthetic peptide by direct ELISA. Clearly this mAb recognises a sequential epitope which is only readily accessible when myoglobins are adsorbed onto a surface (Geysen, 1985).

Studies with monoclonal antibodies directed against the β_2 -subunit of E.coli tryptophan synthase have shown that some antibodies bind rapidly to the native antigen in solution, while others preferentially

recognise the antigen only when it has been adsorbed onto the surface of ELISA plates (Friguet et al, 1984; Djavadi-Ohanian et al, 1984).

Thus antibodies can be induced, during antigen presentation, to epitopes which are inaccessible to antibody when the native antigen is in solution.

1.4.2 Protrusion Index

This method is an extension of accessibility, and takes account of the extent to which a residue protrudes out into the solvent. To evaluate the protrusion of a residue, Thornton et al, 1985, calculated 'equimomental ellipsoids' to fit proteins of known crystallographic structure. A 100% ellipsoid contained 100% of the atoms of the protein within it. A 90% ellipsoid, however, included 90% of the residues, with 10% protruding outside it. Any residue protruding beyond a 90% ellipsoid was assigned a 'protrusion index' (PI) of 9, those outside an 80% ellipsoid assigned PI=8 etc.. Following assignment of PI values to all the amino acids of myoglobin, lysozyme and myohaemerythrin, plots were drawn of PI value versus residue number. A good correlation was found between residue PI maxima, and the locations of amino acids involved in known antigenic sites.

For future use where only the primary sequence is available, Thornton et al have averaged the PI

values of the amino acids found from known structures to give predicted protrusion values for each amino acid.

1.4.3 Hydrophilicity

For water soluble globular proteins to be stable in an aqueous environment, they fold into a structure which tends to bury hydrophobic residues in their interior, and to expose their mostly hydrophilic ones on the surface (Berzofsky, 1985)

In an attempt to find a method to predict the location of antigen sites from primary sequence data, Hopp and Woods (1981) suggested that the most hydrophilic segments of a sequence would correlate well with antigenicity, because these segments are most likely to be on the external surface of a protein. Each amino acid was assigned a numerical value (hydrophilicity index) based on the polarity of its side chain, the data largely coming from the solvent parameters of Levitt, 1976. Repetitively averaging these values for overlapping sets of 6 residues along the chain and plotting them versus the residue number of the leading residue gives a hydrophilicity profile, where a positive value indicates an above average hydrophilicity. Using 12 proteins for which antigenic sites were known, they found that the segment of greatest hydrophilicity for the entire protein was

invariably in one of the known antigenic sites. This approach was then applied to the sequence of the hepatitis B surface antigen in order to identify potential antigenic sites. Following chemical synthesis of the most hydrophilic peptide predicted, it was experimentally shown that this corresponded to a major antigenic determinant of that virus (Hopp and Woods, 1981; Hopp and Woods, 1983). However, the secondary peaks of hydrophilicity do not always correlate well with known antigenic sites. For example, only 2 of the 5 main antigenic sites of myoglobin coincide with hydrophilicity maxima, and some hydrophilicity maxima are not antigenic (Atassi, 1984). For Influenza virus haemagglutinin, a strongly immunogenic region has been reported which is so hydrophobic that the corresponding synthetic peptide is insoluble in aqueous solvents (Atassi and Webster, 1983).

1.4.4 Mobility

Protein molecules in solution do not remain in one set conformation, but due to internal dynamics, parts of the molecule are continually making small conformational changes (Artymuik, 1979; Pain, 1983). Most of the detailed information relating to protein conformational changes have been obtained from highly refined x-ray crystallographic data (Ringe and Petsko, 1985). Present refinements to this technique

not only give precise atomic coordinates, but also atomic temperature factors (B values) (Frauenfelder et al, 1979). The temperature factor represents the mean-square displacement of each atom, and when plotted against residue number gives a graphic image of the degree of mobility existing along the polypeptide chain (Williams and Moore, 1985). Useful information on mobility can also be obtained from nuclear magnetic resonance studies (Moore and Williams, 1980).

Information from these techniques is not readily available at present for many proteins, and mobility predictions are certainly not applicable to cases where only the primary sequence is known (Berzofsky, 1985).

Two different approaches have been used to study the relationship between antigenicity and mobility, the binding of antibodies to peptides and the binding of antibodies to proteins. Antibodies raised against proteins are made against a more conformationally restricted molecule, whereas antibodies raised against peptides have a much less conformationally restricted epitope.

Peptides in solution exist in a large number of conformations in a dynamic equilibrium (Kaiser and Kezdy, 1983). Considering this, a surprisingly high proportion of mAbs raised against peptides react with the intact protein (over 60% in one study of four proteins) (Niman et al, 1983).

Tainer et al, 1984, raised polyclonal

antibodies to 12 peptides, each of between 10-15 residues, covering 70% of the exposed molecular surface area of myohaemerythrin as defined by X-ray crystallography. A very good correlation was found between mobility of segments in the native protein and the ability of antibodies to bind to these sites on the native protein in solution. This correlation was shown to be with the mobility of exposed surface regions, and not just due to surface exposure. However, highly exposed amino acids do tend to be less hydrophobic and due to their external, relatively unconstrained position are more highly mobile than internal residues (Levitt, 1983).

Thus, atomic mobility of a segment of the native protein is a critical factor in determining the ability of antibodies against short peptides corresponding to that segment to bind to the native protein.

A second approach to examine the mobility of antigenic sites was to use antisera against the native protein. The tobacco mosaic virus protein (TMVP) was shown to have 7 continuous antigenic determinants as identified using tryptic peptides (Milton and Van Regenmortel, 1979; Altschuh et al, 1983). When the positions of these epitopes were superimposed on a plot of temperature factors of main chain TMVP atoms, it was found that all seven antigenic regions corresponded to mobility peaks (Westhof et al, 1984). This correlation was better than that between antigenicity and either hydrophilicity or accessibility. Similarly, a good correlation was found between reported continuous

antigenic sites of myoglobin and segmental mobility. These same investigators have concluded that the entire surface of the TMVP is antigenic, although composed primarily of discontinuous epitopes. They suggested that no single criterion can be used to distinguish generally nonantigenic from antigenic regions (Al Moudallal et al, 1985).

One explanation for the apparent correlation found between antibody binding to continuous epitopes, and mobility is an induced fit hypothesis (Tainer et al, 1984; Marx, 1984). In the induced fit model, an initial weak binding is stabilized by a conformational change in the antigen induced by the antibody. In an allosteric model (Berzofsky, 1985), an unfavourable conformational equilibrium is 'pulled' towards the complementary form by trapping of the complementary molecules in a tight complex by the antibody. If the antibody also undergoes a conformational change, a further model can be envisaged (Edmundson et al, 1984).

Geysen, 1985, has suggested how a thermodynamic analysis can be used to explain the interaction between antibody and antigen, allowing for the possibility of conformational changes occurring in both the antibody and the antigen.

1.4.5 Composite Surface Profile

No single parameter has so far been shown

able to predict all the antigenic sites on any protein. Recently a new set of hydrophilicity indices has been derived from the retention times of twenty model synthetic peptides in high performance liquid chromatography (HPLC) (Parker et al, 1986). This group has produced a composite predictive method consisting of HPLC hydrophilicity indices, accessibility (Janin, 1979) and flexibility B-values (Karplus and Schulz, 1985). This composite profile was shown to correlate well with the known antigenic sites for several proteins including myoglobin, lysozyme, cytochrome c and influenza HA1 (Parker et al, 1986)

1.4.6 Structurally Inherent Antigenic Sites

Initially it was thought that immunogenic sites on a protein antigen differ in sequence from their counterpart in the host (Urbanski and Margoliash, 1977). However, it has been found that rabbit or mouse antisera against bovine or human serum albumin exhibit autoreactivity against the animal's own albumin (Atassi et al, 1982). Also rabbits injected with rabbit myoglobin can produce antibodies to it, although use of Freund's complete adjuvant (FCA) is necessary for this to occur. Therefore, a protein need not be foreign to be antigenic. It may be that FCA assists in overcoming tolerance to self-proteins through the by-passing of T cell suppression.

The antigenicity of proteins is conferred on certain surface regions by virtue of the three-dimensional locations of these sites. Therefore it would follow that antigenic sites of conformationally related proteins are likely to have similar molecular locations. Using this concept it has been possible to predict by extrapolation of the three-dimensional locations of the antigenic sites of swMb, many of the sites of human Hb (Karzim and Atassi, 1982) and also those of soybean leghaemoglobin, a distantly related haem protein (Hurrell et al, 1978).

Recently, a computer search has been performed to identify a set of peptide sequences, containing at least six amino acids that occur in unrelated proteins (Wilson et al, 1985). Antibodies are to be raised against these peptides and tested for cross reactivity against them in their different conformations in the various proteins. In this way it will be possible to investigate further the roles of conformation and mobility in antibody binding.

In summary, accessibility, protrusion, hydrophilicity, mobility and inherent conformations may all play a vital role in antigenicity. However, a much larger database of experimental results is needed before the relative importance of each can be defined.

Citrate synthase is a very well characterised protein, as discussed in the following section, and sufficient information is available to apply all the predictive methods, previously discussed, to it.

CHAPTER TWOCitrate Synthase

	Page
2.1 The Citric Acid Cycle	34
2.2 Citrate Synthase	36
2.3 Species Dependent Properties of Citrate Synthase	38
2.4 Primary Sequence Data for Citrate Synthase from Pig Heart, <u>E.coli</u> and Yeast	42
2.5 Physical Structure of Citrate Synthase	45
2.6 Immunochemical Study of Citrate Synthase	48

2.1 The Citric Acid Cycle

The citric acid cycle (fig. 2) operates in most living organisms and has a dual role:

- a) During the degradation of carbohydrates, fats and amino acids it provides reducing equivalents to the electron-transport chain, for energy production in the form of ATP.
- b) It supplies precursors used in gluconeogenesis, lipogenesis, ketogenesis and amino acid biosynthesis.

Even in facultative anaerobes, which lack 2-oxoglutarate dehydrogenase during anaerobic growth, the enzymes of the cycle are able to operate as two arms (citrate → 2-oxoglutarate, oxaloacetate → succinate) and are indispensable for organisms to meet their metabolic requirements.

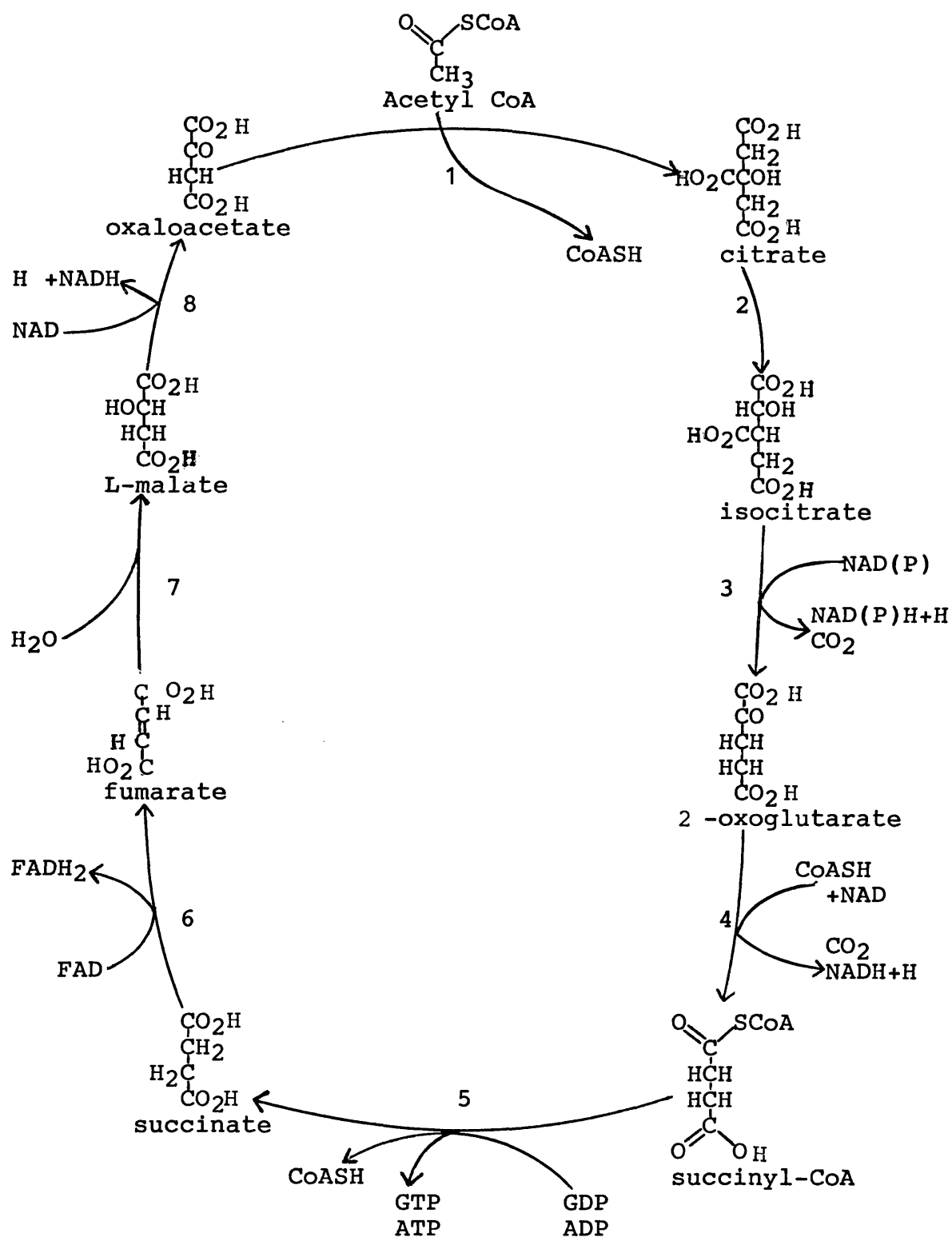
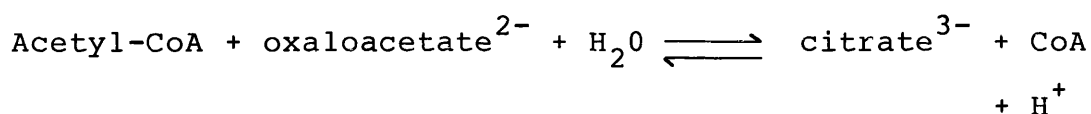


Fig.2 The Citric Acid Cycle. The enzymes are :1-citrate synthase, 2-aconitase, 3-isocitrate dehydrogenase, 4- -oxoglutarate dehydrogenase, 5-succinate thiokinase, 6-succinate dehydrogenase, 7-fumarase, 8-malate dehydrogenase.

2.2 Citrate Synthase

Citrate synthase (citrate oxaloacetate-lyase (CoA acetylating); EC4.1.3.7) catalyses the condensation of oxaloacetate and acetyl CoA (Lynen and Reichert, 1951) in the following reaction,



Due to its key position in the citric acid cycle, citrate synthase has been extensively studied and has been shown to play an important regulatory role in a cell's metabolism (Beeckmans and Kanarek, 1984; Weitzman, 1981; Randle, 1970 and Weitzman and Danson, 1976).

In eukaryotes the citric acid cycle enzymes are exclusively mitochondrial, with the exception of germinating plant seeds where citrate synthase, together with enzymes of the glyoxylate shunt, are also found in glyoxysomes (Tolbert, 1981). Citrate synthase is loosely attached to the inner mitochondrial membrane of eukaryotes (Dsouza and Srere, 1983) and is thought to be associated with the cell membrane of bacteria (Mitchell, 1963). A close association with other enzymes; malate dehydrogenase and fumarase (Beeckmans and Kanarek, 1981), pyruvate dehydrogenase (Sumegi et al, 1980), and aspartate amino-transferase (Fahien and Kmietek, 1983), has also become apparent for citrate synthase. Recent work by Barnes and Weitzman, 1986, has shown by gentle disruption of cells followed by gel filtration

and sucrose density gradient centrifugation, that five sequential enzymes of the citric acid cycle; fumarase, malate dehydrogenase, citrate synthase, aconitase and isocitrate dehydrogenase specifically associate into a cluster.

2.3 Species dependent properties of citrate synthase

Citrate synthase possesses a diversity of subunit structure, catalytic activity and regulation which shows a strong correlation with the taxonomic status of the source organism (Weitzman, 1981; Weitzman and Danson, 1976). A summary of these relationships is shown in table 3.

Studies of eukaryotic, and Gram positive bacterial citrate synthases have shown them to be inhibited by ATP (Jangaard et al, 1968; Hathaway and Atkinson, 1965). This inhibition has been shown to be isosteric, ATP competing with the substrate acetyl-CoA at the active site (Harford and Weitzman, 1975).

Gram negative bacterial citrate synthases have proved insensitive to ATP (Weitzman, 1966), however they are inhibited allosterically by NADH. Within this group, the enzymes of obligate aerobes can be reactivated by AMP, while those of facultative anaerobes cannot (Weitzman, 1981). In Gram positive bacteria and eukaryotes, citrate synthases are generally much less inhibited by NADH, than those of the gram negative bacteria (Weitzman and Danson, 1976).

Inhibition of citrate synthase by 2-oxoglutarate was shown to be allosteric and only occurred in facultatively anaerobic Gram negative bacteria (Weitzman and Dunmore, 1969).

Investigation of the molecular sizes of the citrate synthases of Gram negative bacteria has shown

Table 3 Cross-species properties of citrate synthase.

	EUBACTERIA			ARCHAEBACTERIA			EUKARYOTES
	<u>Gram negative</u> obligate aerobes	<u>Gram negative</u> facultative anaerobes	<u>Gram positive</u>	Halo- philes	Thermo- acido- philes	Methan- ogens	
Molecular weight	270,000	270,000	90,000	110,000	85,000	-	90,000
Type	large	large	small	small	small	-	small
Subunit composition	hexamer	hexamer	dimer	-	dimer	-	dimer
<u>INHIBITION</u>							
NADH	allosteric (reactivated by AMP)	allosteric (AMP has no effect)	none	none	none	yes	v. little (isosteric)
2-oxo- glutarate	none	allosteric	none	none	none	yes	none
ATP	none	none	isosteric	weak	yes	yes	isosteric

them to be 'large', existing as hexamers with approximate molecular weights of 270,000 (Tong and Duckworth, 1975; Robinson et al, 1983a). In contrast, Gram positive bacteria and eukaryotic organisms possess 'small' citrate synthases, which are dimers with total molecular weights of approximately 90,000 (Weitzman, 1981; Robinson et al, 1983b). However, it should be noted that the subunit sizes in both the 'large' and 'small' type enzymes are similar.

Archaeobacteria consisting of halophiles, thermoacidophiles and methanogens are proposed to constitute a third evolutionary line of descent (Woese, 1981). Of those studied to date, the citrate synthases of the thermoacidophiles have been shown to be dimers of total molecular weight 85,000. They are sensitive to inhibition by ATP (Danson et al, 1985). Halophiles also have the small type of citrate synthase and are inhibited only weakly by ATP. Citrate synthase from the methanogen Methanosarcina barkeri was unusually inhibited by ATP, NADH and 2-oxoglutarate; however the enzyme is present in very low amounts and therefore its molecular size has not yet been determined (Danson et al, 1985).

In summary, allosteric regulation is seen only in 'large' citrate synthases, the regulation of the 'small' enzymes being isosteric.

A complementary approach has been used to study the structure/function relationships of citrate synthases. This was achieved using the facultative

anaerobe Escherichia coli. The 'wild type' of this organism produces a 'large' citrate synthase, of molecular weight 270,000, which is sensitive to allosteric inhibition by both NADH and 2-oxoglutarate (Weitzman and Danson, 1976). From mutagenesis work, a mutant of E.coli was produced, whose citrate synthase was small, of molecular weight 100,000, and was insensitive to inhibition by either NADH or 2-oxoglutarate (Danson et al, 1979). The apparently minor genetic alterations involved in this conversion emphasises the strong relationship between the structure and function of citrate synthase, and may mimic natural mutations which have occurred to produce the existing diversity of citrate synthases.

2.4 Primary sequence data for citrate synthase from pig heart, E.coli and yeast

Amino acid sequencing performed on fragments of pig heart citrate synthase (PHCS) produced by proteolytic (Bloxham et al, 1980) and chemical means, has provided the complete amino acid sequence of this enzyme (Bloxham et al, 1981; Bloxham et al, 1982), see fig. 3.

Primary sequence data for citrate synthase from E.coli came first from sequencing the gene (Ner et al, 1983). Amino acid sequencing performed on fragments produced by proteolytic and chemical digests of E.coli citrate synthase have since confirmed the majority of this sequence.

The nucleotide sequence of citrate synthase from yeast has also been determined by Suissa et al, 1984. The amino acid sequence, derived from the nucleotide sequence shows that this citrate synthase has a strongly basic amino-terminal region, fig. 3. It has been proposed that this amino-terminal region is removed during translocation of the precursor into mitochondria.

From figure 3, it can be seen that a substantial degree of sequence homology exists between the citrate synthases of pig heart and yeast (Garforth, 1986). Citrate synthase from E.coli does show regions of homology with both the citrate synthases from pig heart, and yeast, but this is not as great as that


```

PCS                               ASSTNLKDILADLI10
YCS      MSAILSTTSKSFSLRGSTRQCQNMQKALFALLNARHYSSASEOTLKERFAEII
ECS                               A-DIKAKLTLDGDT

      20      30      40      50      60      70
PKEQARIKTFRQOHGNTVVGQITVD-MMYGGMRGMKGLVYE-TSVLDPDEGIRFRGYSI
PAKAQEIKKFKKEHGKTVIGEVLLLEQAYGGMRGTKGLVWE-GSVLDPDEGIRFRGRTI
AVELDVLKGTLCQDVIDIRTLGSKGVFTFDPGFTSTASCESKITFIDGDEGI-----

      80      90      100     110     120     130
PECKMLPKAKGGBEPLPEGLFWLLVTGQIPTEEQVSWLSKEWAKRAALPSHVVTMLDN
PEIORELPKAEGSTPEPLPEALFWLLLTGEIPTDAQVKALSADLAARSEIPEHVIQLLDS
LLHRGFPIDQLATDSNYLE-VCYILLNGEKPTDEQYDEFKTTVTRHTMIHEQITRLFHA

      140     150     160     170     180
FPTNLHPMSQLSAAITAINSESNFARAYAEGLHRTKYWELIYEDCMOLIAKLFCVAAKI
LPKDLHPMAOFSTIAVTALESSEKFAKAYADGVSKKEYWSYTFEDSLDLGKLPVIAISKI
FRRDSHPMAVMGGITGALAAFYHDSLDVNNPRHREI-----AAFRLLSKMPMTMAAMC

190      200     210     220     230     240
YRNLRYR----EGSSIGAIIDSKLDWSHNFNMLGYTD-AQFTFLMRLYLTIHSDHEGGNV
YRNVFK----DGK-IITSDPNADYGKNLAQLLGYE-NKDEIDLMRLYLTIHSDHEGGNV
YKYSIGQPFVYPRNDLSYAGNFLNMMFSTPCEFYEVNPILERAMDRTILTIHADHEQ-NA

      250     260     270     280     290     300
SAHTSHLVGSALS DPYLSFAAAMNGLAGPLHGLANQEVIVWLTQLOKEVGKQVSDKLR
SAHTSHLVGSALS SPYLSLAAGLNGLAGPLHGRANQEVLEWLFKLREEVKGQDYSKETIE
STSTVVRTAGSSGANPRACIAAGIASLWGHAGGGANEAAKML-EEISSVKHIPEFPRRA

      310     320     330     340     350     360
DYIMNTLNSGRVVPGYGHAVLRKTDPRYTQORE-FALKHLLHDPMFKLVAQLMKIVENV
KYLWDTLNA GRVVPGYGHAVLRKTDPRYTAORE-FALKHLLDYELFKLMSTIYEVAEGV
KDKND----SFRLMGFGHRVYKNYDPRATVMRETCHVLKELGTDKDDILEVAMELENIA

      370     380     390     400     410
LL-EQGAKNPWPNVDAHSGVLLQYYGMTEFNYYTVLFGVSRALGVLAQLIWSRALGFF
L-TKHGKTKNPWPNVDSHSGVLLQYYGLTEASFYTVLFGVARAIGVLPOLIIDRAMGAP
LNDPYFIEKKLYPNVDFYSGIILKAMGIP-SSMFTVIFAMARTVGWIAHWSEMHSDDMK

420      430
LERPKSMSTDGLIKLV--DSK
LERPKSFSTEKYKELVKKIESKN
LARPRQLYTGYEKRDFK-SDIKR

```

Fig. 3.

Comparison between the amino acid sequences of citrate synthase from pig heart (PCS), yeast (YCS) and *E. coli* (ECS). The sequences are aligned to maximise homologies. Regions of homology are boxed. Adapted from Suissa *et al* 1984. Residues are numbered according to the sequence of PHCS.

between the pig heart and yeast enzymes. Residues shown to be essential for catalysis in the PHCS are highly homologous in both the yeast and E.coli enzymes. These include His235, His238, His274, His320, Arg329 and Asp375 (Suissa et al, 1984).

The availability of primary sequence data makes citrate synthase an ideal enzyme on which to perform epitope mapping studies. The relevant limited proteolysis and chemical fragmentation work performed during the sequencing and verification of the primary sequence of PHCS is discussed further in section 6.0.

2.5 Physical Structure of Citrate Synthase

An extensive crystallographic study has been performed on pig heart citrate synthase and minor work on chicken citrate synthase (Wiegand et al 1979; Remington et al, 1982). For the pig heart enzyme, the conformation has been shown to contain 40 helices per dimer, packing tightly to form a globular molecule. Each monomer is divided structurally into two domains, see figure 4. The larger domain contains residues 1-274 (helices A to M) and residues 381-437 (helices S and T), while the smaller domain has residues 275-380 (helices N to R). The subunits are extensively interdigitated, with amino acid residues from one subunit contributing to the binding of citrate and CoA to the other subunit.

Kinetic studies, by Johansson and Pettersson (1977) have indicated that the interaction of citrate synthase with its substrates follows an ordered mechanism. Initial binding of oxaloacetate increases the ability of acetyl-CoA to bind to the enzyme.

The structure of the enzyme has been described in three states (Remington et al, 1982; Wiegand et al, 1984). It has been proposed that binding of oxaloacetate to the 'open' form of the enzyme induces a 'closed' form in which the binding site for coenzyme A is developed. A further 'closed' form is found which has bound citrate. The conformational changes in response to changes in the state of ligation have been

PIG HEART CITRATE SYNTHASE

(monomeric form)

A	5 - 29	K	221 - 236
B	37 - 43	L	242 - 255
C	70 - 78	M	257 - 271
D	88 - 99	N	274 - 291
E	103 - 118	O	297 - 312
F	121 - 131	P	327 - 341
G	136 - 152	Q	344 - 365
H	153 - 161	R	373 - 386
I	163 - 195	S	390 - 416
J	208 - 218	T	426 - 433

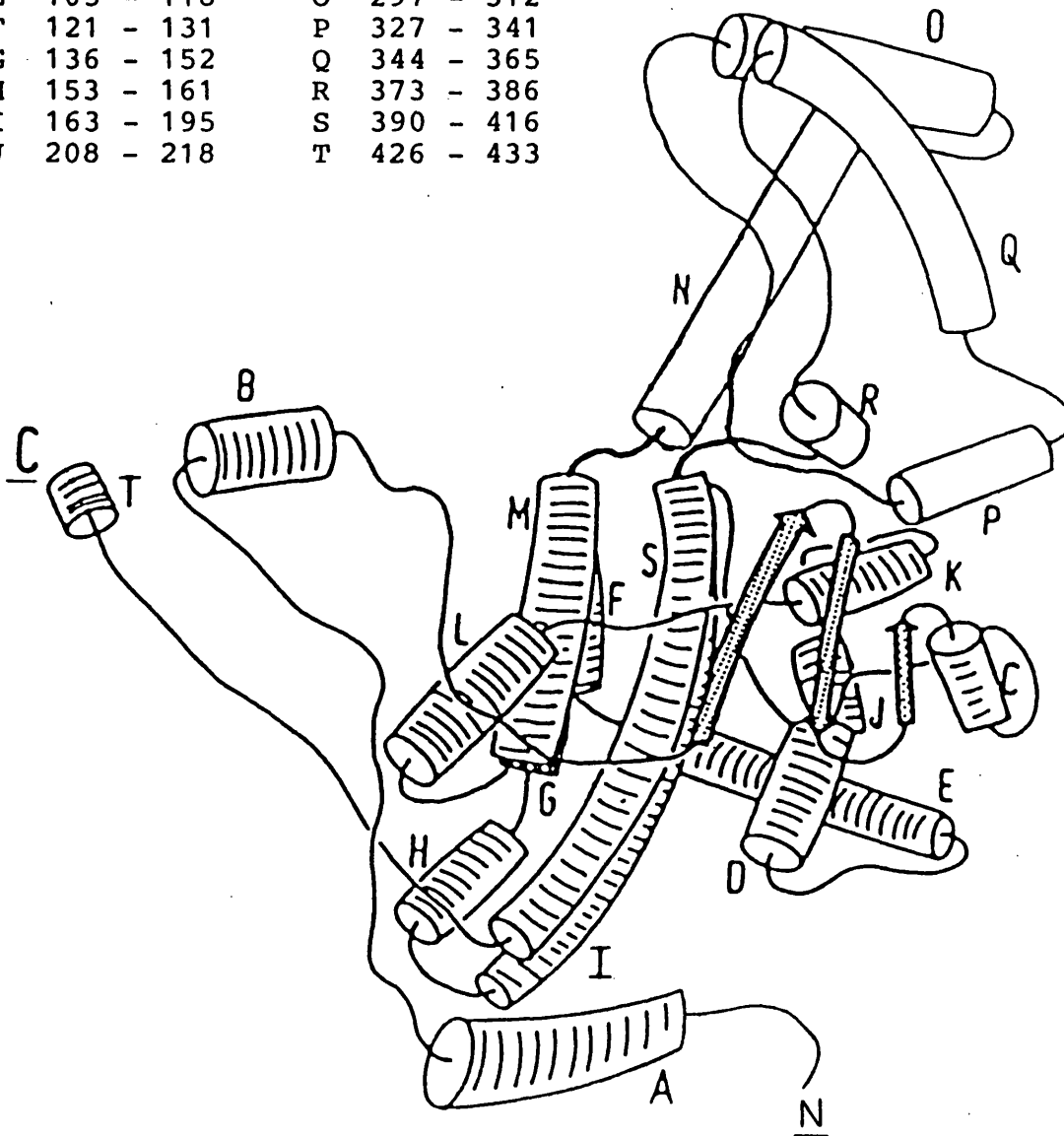


Fig.4 Schematic representation of the structure
of PHCS

studied by another group of workers (Lesk and Chothia, 1984; Chothia and Lesk, 1985). The conformational forms of the enzyme were explained in terms of helix movements, the most pronounced occurring of helix 0 on the outside of the small domain. This is in agreement with the proposal of Wiegand et al 1984, who suggested that the small domain is more flexible than the large domain.

2.6 Immunochemical Study of Citrate Synthase

Moriyama and Srere, 1971, showed by Ouchterlony double diffusion that an antiserum raised against rat heart citrate synthase was able to cross-react with rat liver, kidney, brain and spleen enzymes. However, no reaction was seen with PHCS, or CS from moth muscle, Azotobacter, or mango. Further experiments using this antiserum for enzyme precipitation and in double immunodiffusion experiments suggested that the rat kidney CS, heart CS and brain CS are immunologically identical (Matsuoka and Srere, 1973). However the insensitivity of the double immunodiffusion method must be emphasised in drawing such conclusions.

Other antisera raised against yeast CS have proved useful for precipitation of the translation products of mRNAs, to identify which mRNAs could be used to produce cDNA sequences coding for CS (Suissa et al, 1984; Alam et al, 1982).

In an attempt to discover which domain of PHCS had the greater antigenicity double diffusion assays and immunoprecipitation experiments were performed on various proteolytic digest mixtures of PHCS (Bloxham et al, 1980). The relative impurities of the fragments used in this assay, however, may overrule the conclusion that the small domain is the primary antigenic domain.

The ability to use rabbit antibodies raised against PHCS in an immunochemical investigation of interspecies diversity was shown by Pullen et al, 1985. In a competition enzyme-linked immunosorbent

assay, where plates were coated with pigeon breast CS, they showed the order of competition to be PHCS > pigeon breast CS > Bacillus megaterium CS > E.coli CS. The same order of reactivity was shown in enzyme inhibition experiments. In this experiment, however, the antibodies appeared able to activate the citrate synthase from E.coli. Finally, cross-reactivity was studied by examining the ability of the rabbit anti-PHCS immunoglobulins to precipitate out enzyme activity from solution using Staphylococcus aureus protein A as a cross-linking agent. In this assay both the PHCS and E.coli CS tested were shown to be precipitated. This work shows conclusively that citrate synthases from pig heart, pigeon breast, B.megaterium and E.coli share common antigenic determinants.

This work confirms the feasibility of using immunochemical methods to investigate the structural diversity of citrate synthase and provides the basis for the application of monoclonal antibodies.

Aims of this Study

The work described in this thesis is designed:

1. to raise, using different screening methods, a library of mAbs specific for PHCS
2. to investigate the potential of various immunochemical screening methods for future use
3. to characterise the binding of mAbs to PHCS using epitope analysis of fragments of PHCS
4. to compare the experimentally determined epitopes of PHCS with those predicted by four different computer aided model systems

CHAPTER THREEMATERIALS AND METHODS

	Page
<u>MATERIALS</u>	54
3.1 Cells and cell culture materials	54
3.2 General reagents and solutions	55
<u>METHODS</u>	56
<u>Cell Culture</u>	56
3.3 Maintenance of cell lines	56
3.4 Cell counting	56
3.5 Cryopreservation of cells	57
3.6 Preparation of mouse peritoneal feeder cells for hybridoma growth	57
3.7 Isolation of immune spleen cells	58
3.8 Fusions	58
3.9 Cloning of hybrids	60
3.10 Production of ascitic fluid	60
3.11 Preparation of HEp-2 slides	60
3.12 Immunofluorescence staining of fixed HEp-2 slides	61
<u>Antibody preparation</u>	62
3.13 Immunisation procedures	62
3.14 Preparation of sera	63
3.15 Immunoglobulin purification from sera	63

3.16	Monoclonal antibody purification from ascitic fluid	64
3.17	Protein determination	65
	<u>Screening methods</u>	66
3.18	Antigen coating for ELISAs	66
3.19	ELISA for anti-citrate synthase	68
3.20	Antigen coating for immunodot blot assay	69
3.21	Immunodot blot assay for anti-citrate synthase	69
3.22	Antibody subclass typing	70
	<u>Electrophoretic and immunoblotting techniques</u>	70
3.23	Gradient SDS-polyacrylamide gel electrophoresis	70
3.24	Sample preparation for SDS-PAGE	75
3.25	Electrophoretic transfer of proteins from PAGE gels to nitrocellulose	76
3.26	Passive transfer of proteins from PAGE gels to nitrocellulose	77
3.27	Staining polyacrylamide gels	78
3.28	Amido black staining of nitrocellulose bound protein	78
3.29	Immunoblotting of nitrocellulose bound proteins	78
	<u>Fragmentation of PHCS</u>	79
3.30	Limited proteolysis of PHCS	79
3.31	Chemical fragmentation of PHCS	80

3.32	Preparation of mAb immunosorbent columns	81
3.33	MAb immunosorbent chromatography	82
	<u>Enzyme inhibition studies</u>	82
3.34	Assay of citrate synthase activity	82

MATERIALS

3.1 Cells and cell culture material

The HAT sensitive mouse myeloma X63-Ag8.653 and the human epithelial cell line HEP-2 were obtained from Flow Laboratories, Scotland.

Sterile media, supplements and plasticware were obtained from Nunc Gibco, Flow Laboratories and Sterilin.

All PBS used for cell culture was prepared from tablets obtained from Flow Laboratories, PBS Dulbeccos formula (modified) calcium and magnesium free. The trypsin used was Type X1 from bovine pancreas, salt free, Sigma T-1005.

Complete RPMI contained RPMI 1640, 4mM glutamine, 50 μ M 2-mercaptoethanol (Sigma), 10% (v/v) 'myoclone' foetal calf serum (FCS) and 50 μ gml⁻¹ kanamycin. Tylocine at 60 μ gml⁻¹ was sometimes used in place of kanamycin.

Complete BME contained BME (modified) with Earle's salts, 4mM glutamine, 10% (v/v) FCS or newborn calf serum (NCS) and 50 μ gml⁻¹ kanamycin.

Freezing medium contained 50% (v/v) myoclone FCS, 20% (v/v) DMSO, 30% (v/v) complete medium and was stored at -20°C.

45% Polyethylene glycol (PEG) solution was prepared by autoclaving 4.5g PEG 4000 (GK), from Merck, with 5ml PBS. 0.5ml of dimethyl sulphoxide

(DMSO) was added after cooling and it was found that the solution could be stored for up to 1 month at room temperature.

3.2 General reagents and solutions

All chemicals used came from Sigma or BDH, and were of Analar or equivalent grade except palmitoyl CoA, which was a gift from Dr. T. Else. All enzymes were purchased from Sigma, or Boehringer Mannheim. Subtilisin BPN', protease type VII was Sigma P-5255, chymotrypsin type II from bovine pancreas was Sigma C-4129, and trypsin type III-S from bovine pancreas was Sigma T-2395. Immunochemical reagents were from Sigma, unless stated. Chromatography media were obtained from Pharmacia.

The solutions were prepared using glass distilled water. Buffers were made as described by Hudson and Hay 1980, or Dawson et al, 1974, or derived from them.

METHODS

Cell Culture

3.3 Maintenance of cell lines

Mouse myelomas (X63-Ag8.653) and established hybridomas, were grown at 37°C in complete RPMI in an atmosphere of 3.5% CO₂ in air with a relative humidity of 95%. Under these conditions the cell lines had doubling times of 18-24h, and were maintained in log phase growth. The HAT sensitivity of myelomas was maintained by periodic inclusion of 0.13mM 8-azaguanine in the culture medium.

The human epithelial cell line HEp-2 was grown at 37°C, in monolayer culture in complete B.M.E., in an atmosphere of 5% CO₂ in air with a relative humidity of 95%. When cells had grown to confluency, they were harvested by trypsinisation and new cultures initiated at a split ratio of 1:40.

For trypsinisation, cells were washed with PBS and incubated for 1 min at 37°C with trypsin (1ml of 0.05% trypsin in PBS/5cm² area). The trypsin was decanted and the flask closed and incubated at 37°C for 15 min. Cells were resuspended in medium prior to sub-culturing.

3.4 Cell counting

For a total cell count, equal volumes of cell suspension and Turk's solution (10% (w/v)

gentian violet, 3% (v/v) glacial acetic acid) were mixed and incubated for 5 min at room temperature. All cells are stained by this method.

For a viability count, equal volumes of cell suspension and nigrosine (0.2% (w/v) in PBS) were mixed and incubated for 5 min at room temperature. Only dead cells are stained by this method.

In both cases, cell counting was performed using a haemocytometer with improved Neubauer ruling.

3.5 Cryopreservation of cells

Up to 5×10^6 cells were pelleted by centrifugation at 200g for 5 min, and resuspended in 0.5ml complete medium. The suspension was transferred to a 2ml cryopreservation tube, and cooled on ice for 15 min, prior to the addition of an equal volume of cold freezing medium. Ampoules were placed in the vapour phase of liquid nitrogen to freeze at a rate of -1°C per min, then stored in liquid phase nitrogen until required. Frozen cells were recovered by rapid thawing to 37°C , followed by washing with PBS and resuspension in complete medium.

3.6 Preparation of mouse peritoneal feeder cells, for hybridoma growth

Adult BALB/c mice were killed by cervical dislocation and were then washed in 70% ethanol. The abdominal skin was removed and 5ml of sterile

PBS was injected peritoneally through a 20G needle. After gentle massage of the abdomen, the peritoneal cavity was cut open just enough to allow the removal of fluid using a pasteur pipette.

The cells were irradiated with 2000 rads of γ -radiation from a cobalt source, and pelleted by centrifugation for 5 min at 200g. Following resuspension in complete medium, or HAT medium, a total cell count was performed.

Feeder cells were used to a final concentration of 1×10^5 cells/ml, whenever a new 4 well or 96 well tray was used for cloning or cell transfer. Feeder cells were also included in flasks when a low cell density culture was to be accelerated into rapid cell growth.

3.7 Isolation of immune spleen cells

Primed adult BALB/c mice were boosted, then killed 4 days later, and their spleens removed aseptically. Spleen cells were perfused with 10ml of PBS, in a 90mm petri dish. Each spleen yielded approximately 1×10^8 cells.

3.8 Fusions

Myeloma cells (1×10^7), with a minimum viability of 90%, were transferred to a 50ml, sterile, siliconised glass centrifuge tube. Spleen cells (1×10^8) were transferred to the same tube, care

being taken to exclude any pieces of spleen sack. Cells were pelleted by centrifugation for 5-10 min at 200g, and the supernatant was carefully discarded. After loosening the pellet, 1ml of PEG solution was added dropwise to the pellet with continuous agitation. The tube was incubated in a 37°C water bath, with continuous shaking for 90s, after which the following additions of PBS were made: 1ml over the first 30s, 3ml over the next 30s, and 16ml over the next minute. A further 3-5 min incubation at 37°C preceded the cells being pelleted by centrifugation and resuspended in HAT medium.

One fusion, as above, would be plated out into five 96 well microtitre trays. Alternatively, the cells were resuspended in complete medium, grown in culture for 24 h, and frozen down into 5 cryotubes.

For the first 3 weeks of growth after fusion, cells were grown in HAT medium, changed at least twice. Prior to transfer to complete medium, cells were grown for 3 weeks in HT medium which was changed at least twice to overcome the toxicity of aminopterin alone in the medium.

Cells were screened for the presence of anti-PHCS antibodies when they were half confluent in the 96 well dishes, approximately 2-3 weeks after fusion, or after cloning.

3.9 Cloning of hybrids

Hybrids were cloned by single cell transfer (Gagnon and Raymond, 1985) using a micromanipulator and microscope. Cells were suspended in 2ml medium in a 50mm petri dish, to a concentration at which only 1-2 cells were visible under the high power lens (magnification of 200 x) of an inverted phase microscope. For each manouvre, a single cell was sucked up into a medium reservoir contained in a drawn out Pasteur pipette using the micromanipulator and transferred to one well of a 96 well tray, pre-seeded with feeder cells.

3.10 Production of ascitic fluid

Adult BALB/c mice were primed by intraperitoneal injection of 0.5ml pristane (2, 6, 10, 14-tetramethylpentadecane). Hybrid cells (3×10^5 in PBS) were injected intraperitoneally 7-14 days after pristane priming. When visible growth was observed, 1-3 weeks later, the peritoneal cavity was drained every two days for 1 week until sacrificing (Brodeur et al, 1984).

3.11 Preparation of HEp-2 slides

Five sterile multitest slides were placed in the bottom of a square 120mm sterile petri dish and covered with 20ml of complete B.M.E..HEp-2 cells (20ml at a concentration of 5×10^4 cells ml⁻¹

in complete B.M.E.) were dispersed into the petri dish and incubated at 37°C, until cells were approximately half confluent on the slides.

Slides were transferred into a Coplin jar, and washed twice for 10 min in PBS. Cells were fixed by incubating slides for 1 min in ice cold acetone followed by 5 min in a further change of ice cold acetone. Slides were then allowed to dry in air. Fixed slides were stored desiccated at -20°C until required.

3.12 Immunofluorescence staining of fixed HEp-2 slides

The method used was adapted from Johnstone and Thorpe (1982).

Prior to use, slides were washed twice for 10 min with PBS. Blocking was performed by washing twice in 20 min with either normal goat serum diluted 1/10 with 1% casein in PBS, or 1% casein in PBS. Excess fluid was removed by using a cotton-tipped applicator to dry between all wells.

Monoclonal antibodies to be tested were used at a concentration of $100\mu\text{gml}^{-1}$, and sera were used at a 1/40 dilution in PBS. 30 μl of diluted test sample were applied to each well of a fixed multitest slide, which was then incubated at 37°C for 30 min. Slides were then washed in three changes of PBS, each for 5 min, and again dried between wells using a cotton applicator. To each well, 20 μl

of polyvalent goat anti-mouse immunoglobulin-FITC conjugate (Sigma), diluted 1:40 in PBS, were added. Slides were incubated in a dark humidified chamber for 30 min at room temperature, and again washed three times in PBS. Finally, to diminish background fluorescence, slides were counterstained with 0.01% Evan's blue dye, for 5 min.

The slides were mounted by adding 5 μ l of mounting fluid to each well, and covering with a 24 x 62mm cover slip. Mounting fluid was 0.1% para-phenylenediamine, 10% glycerol adjusted to pH8.0 with 0.2M bicarbonate buffer pH9.0, and was stored at -20°C.

Following examination under a Zeiss microscope D-7082 fitted with a UV microscope illuminator-100, slides were stored at -20°C.

Antibody Preparation

3.13 Immunisation procedures

(a) Mice: Adult BALB/c mice were injected with 200 μ l of immunogen (50 μ g PHCS, 100 μ l Freund's complete adjuvant (FCA), made up to 200 μ l with azide free PBS), intraperitoneally, or subcutaneously. Booster injections of 200 μ l immunogen (50 μ g PHCS in 200 μ l azide free PBS) were given intraperitoneally at intervals of 7 days or greater.

(b) Rabbits: Intramuscular injections of

0.5ml immunogen (200µg protein in 50% FCA, 50% azide free PBS) were given in the hind limbs. Booster injections, 0.5ml, of 200µg protein in azide free PBS were given at intervals of 10 days or greater.

3.14 Preparation of sera

(a) Mice: Immunized mice, 7 days after an antigen boost, were either tail bled (for 20µl blood), or exsanguinated by cardiac puncture (for up to 1ml blood).

(b) Rabbits: Immunized rabbits, 9-10 days after an antigen boost, were ear bled for 10ml blood.

Blood was clotted for 1h at 37°C, and clots removed by centrifugation (in a bench MSE centrifuge) at 3,000rpm for 20 min.

Serum was stored at -20°C, either aliquotted, or in 50% glycerol.

3.15 Immunoglobulin purification from sera

To rabbit serum, or mouse serum, saturated ammonium sulphate solution was added to give a 50% saturated solution. Following a 30 min incubation at 4°C, with periodic stirring, the precipitate was collected by centrifugation at 5,000g for 20 min. The pellet was redissolved in water, and further saturated ammonium sulphate solution added to give a 50% saturated solution. Following incubation and centrifugation, as before, the pellet was redissolved in a minimal volume of 30mM phosphate buffer pH8.0,

and dialysed against this buffer at 4°C.

Further purification was performed using ion-exchange chromatography. DEAE-cellulose (1g wet weight for every ml serum) was packed into a column (1.8cm diameter) following equilibration with 30mM phosphate buffer, pH8.0. The dialysed crude immunoglobulin preparation was loaded onto the column and eluted with 30mM phosphate buffer, pH8.0, at a flow rate of 25mlh⁻¹. Fractions were collected until all the protein (immunoglobulin) fraction was eluted. Aliquots were stored at -20°C.

Columns were washed with 0.5M NaCl, and reequilibrated for further use in 30mM phosphate buffer, pH8.0.

3.16 Monoclonal antibody production from ascitic fluid

Freshly collected ascitic fluid was clotted for 1h at 37°C, and clots removed by centrifugation (in a bench MSE centrifuge) at 3,000rpm for 20 min. Monoclonal antibodies were purified by ammonium sulphate precipitation as described in section 3.13. The pellet was dissolved in a minimum volume of PBS and dialysed against this buffer at 4°C. MAb preparations were stored at -20°C, in 50% glycerol.

Further purification was performed using affinity chromatography on immobilized Protein A by the method of Johnstone and Thorpe, 1982. Protein A coupled to Sepharose CL-4B (Pharmacia), at a

concentration of 2mg protein per ml of gel, was packed into the column. Prior to use the column was washed stepwise with 0.1M sodium phosphate buffer, pH8.0, 0.1M citric acid and 0.1M sodium phosphate buffer, pH8.0.

The pellet collected following ammonium sulphate precipitation of crude ascitic fluid was dissolved in 0.1M sodium phosphate buffer, pH8.0, and dialysed against this same buffer. The dialysate was loaded onto the column at approximately 2mlh^{-1} , (the equivalent of 1ml ascitic fluid was loaded per 2ml of coupled Sepharose). The absorbance of the eluant was monitored at 280nm, and 2ml fractions were collected. The column was extensively washed with 0.1M phosphate, pH8.0. Elution was performed batchwise with; 0.1M sodium citrate, pH6.0, to obtain IgG1, 0.1M sodium citrate, pH4.5, to obtain IgG2a, and 0.1M sodium citrate, pH3.5, to obtain IgG2b. Protein containing fractions were dialysed against PBS, and pure mAbs were stored at -20°C in 50% glycerol.

Finally, the column was washed with 0.1M citric acid and reequilibrated in 0.1M sodium phosphate, pH8.0.

3.17 Protein determination

Protein concentrations were determined using the Coomassie brilliant blue dye-binding assay (Bradford, 1976). To 1-20 μg of protein in a volume of 100 μl was added 1ml of colour reagent (10% (w/v)

Coomassie brilliant blue G, 4.75% (v/v) ethanol, 8.5% (w/v) phosphoric acid). The colour reagent (stored at room temperature in the dark) was filtered prior to use. After 10 min-1h the absorbance of the sample under test was read at 595nm. For each assay, a fresh standard curve of Abs vs concentration (1-20µg in 100µl) of standard protein was prepared from which to read off the concentration of the protein under test. Ovalbumin ($E_{1\text{cm}}^{1\%} = 7.35$ at 280nm) was used as the standard protein.

Screening Methods

3.18 Antigen coating for ELISAs

ELISA reactions were carried out using flat-bottomed (rigid polystyrene) EIA strips (Titertek, Flow Lab.). Unless otherwise stated, PBS was used as incubation and washing buffer throughout. All conditions were optimized for each assay, details of which are summarized in Fig. 4. For assays I, II and III wells were coated for 18h at 4°C using a solution of PHCS ($10\mu\text{gml}^{-1}$) in 0.05M sodium carbonate (pH9.8), PBS (pH7.3) or 0.1M sodium acetate (pH4.2) buffer respectively. Plates were then blocked by incubation for 30 min at 37°C with 1% (w/v) casein in PBS and washed with PBS.

Poly-L-aspartate (assays IV and V) or poly-L-lysine (assays VI and VII) coated plates

ANTIGEN COATING

ANTIBODY DETECTION

Direct coating of PHCS at:

I,IX-pH 9.8
II,X-pH 7.3
III,XI-pH 4.2

Poly-aspartate pre-coated
PHCS coated at:

IV-pH 9.8
V-pH 7.3

Poly-lysine pre-coated
PHCS coated at:

VI-pH 4.2
VII-pH 7.3

Rabbit IgG anti-PHCS pre-coated
PHCS coated at:

VIII,XII-pH 7.3

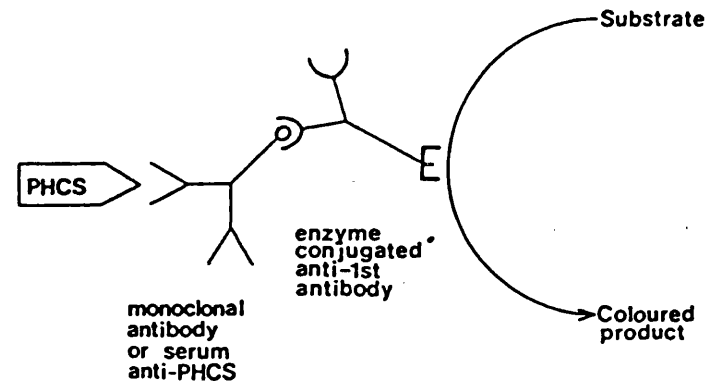
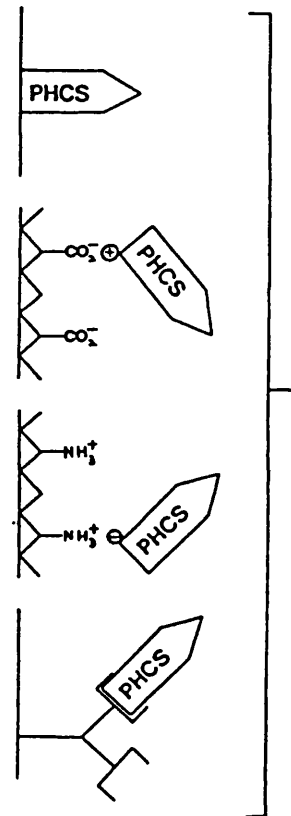


Fig 4 Summary of assays I-XII

were prepared using $50\mu\text{gml}^{-1}$ solutions of polymer incubated for 30 min at 37°C . After washing, citrate synthase was bound at 37°C for 1h using a $10\mu\text{gml}^{-1}$ solution in PBS (assays V and VII) or carbonate buffer (assay IV) or a $5\mu\text{gml}^{-1}$ solution in acetate buffer (assay VI). The plates were then blocked with PBS + 1% casein and washed as above.

Plates for the sandwich ELISA (assay VII) were coated for 18h at 4°C with rabbit IgG anti-citrate synthase ($10\mu\text{g}$ protein/ml in carbonate buffer), blocked with PBS + 1% casein and washed. Antibody coated wells were then incubated with citrate synthase ($10\mu\text{g}/\text{ml}$ in PBS) for 1h at 37°C and washed.

3.19 ELISA for anti-citrate synthase

Purified mAb, culture supernatants, or antisera at appropriate dilution were incubated in antigen-coated plates for 1h at 37°C . The amounts of mAb or antiserum were selected from dilution curves and represent values at which all mAbs gave a positive response when compared with a normal mouse serum control. After washing, alkaline phosphatase-conjugated goat anti-mouse IgG (diluted 1|1000) was added for 1h at 37°C followed by further washing and addition of substrate (p-nitrophenyl phosphate, $1\text{mg}/\text{ml}$ in 10% diethanolamine-HCl buffer, pH9.8). Incubation with substrate at room temperature was continued until a standard test serum (mouse anti-citrate synthase) reached maximum absorbance ($A_{405} = 1.5$),

at which time all reactions were stopped by addition of NaOH and A_{405} values determined using a Titertek Uniskan plate reader.

3.20 Antigen coating for immunodot blot assay

All microculture plates were pre-coated with PBS + 1% casein, which was also used as incubation and blocking buffer unless otherwise stated. Circles (5mm diameter) were cut from nitrocellulose filters (0.2 μ m pore size, Schleicher and Schuell), and one circle was placed in each well of a microculture plate. Citrate synthase solution (40 μ l, 100 μ g/ml buffer) was added to the wells and allowed to dry onto the nitrocellulose for 18h at 37°C. The nitrocellulose was then blocked for 2h at 37°C and washed. Antigen was coated in carbonate buffer for assay IX, PBS for assay X, or acetate for assay XI. For the sandwich blot (assay XII), PBS (40 μ l) containing rabbit IgG anti-citrate synthase (100 μ g/ml) was dried onto nitrocellulose, which was then blocked and coated with citrate synthase (10 μ g/ml in PBS) as above.

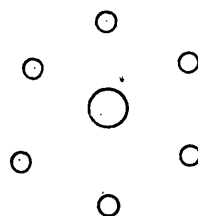
3.21 Immunodot blot assay for anti-citrate synthase

Antigen-coated nitrocellulose circles were incubated, for 18h at 4°C, with the appropriate dilutions of purified mAb, culture supernatants or antiserum. After washing, the circles were then incubated with conjugate for 18h at 4°C followed

by substrate for 35 min at room temperature. At this time a sample (150 μ l) of the supernatant was removed to a clean microplate for measurement of A_{405} as described above.

3.22 Antibody sub-class typing

A commercial (Serotec) ouchterlony typing method was employed to establish the sub-class of each monoclonal antibody. The kit consisted of an agar plate with pre-bored wells, as shown, together with sheep anti-mouse immunoglobulins (IgG1, IgG2a, IgG2b, IgG3, IgA, IgM).



75 μ l of test culture supernatant, or diluted ascitic fluid, was placed in the centre well. 10 μ l of each of the six antisera was dispensed into the outer 6 wells. The plate was tightly closed, and stored flat at room temperature, for 24-48h. After this time, the immunoprecipitate relevant to the immunoglobulin specificity was visible.

Electrophoretic and immunoblotting techniques

3.23 Gradient SDS-polyacrylamide gel electrophoresis

All electrophoreses were performed using a Studier-type slab gel apparatus assembled according to Hames and Rickwood, 1981.

i) Laemmli method (Laemmli, U.K., 1970).

A discontinuous buffer system, containing 0.1% SDS, was used. The final concentration of buffers was: 0.125M Tris-HCl, pH6.8, in the stacking gel, 0.375M Tris-HCl, pH8.8, in the resolving gel, and 0.025M Tris, 0.192M glycine, pH8.3, in the reservoir buffer.

The resolving gel mixtures were made up according to table 3a, for either 8-12%, or 8-20% gradients, and degased prior to addition of TEMED. 17.5ml of the required gradient mixtures were dispensed into a linear gradient maker (LKB, total capacity 40ml) fitted to a peristaltic pump. Using a magnetic stirrer for mixing, and a flow rate of 3-5ml/min, the gradient resolving gel was poured vertically from the top of the gel. Final delivery to the gel was through a very thin tube pushed to the bottom, centre, of the gel, and gradually retracted to keep it above the filling gel surface. 1ml of water saturated isobutanol was layered onto the gel, and left for 30 min, for polymerization to occur. The gel was overlayed with 0.375M Tris-HCl, pH8.8, and left overnight, before the stacking gel was loaded.

All Tris buffer, and remaining isobutanol, were removed from the gel surface. The stacking gel mixture was made up according to the recipe in table 3a and degased prior to the addition of TEMED.

Following insertion of the comb into the glass plates, the stacking gel was loaded using a Pasteur pipette, and allowed to set. Once set, the comb was removed, and the gel transferred to the running apparatus (Hames and Rickwood, 1981), with 400ml of buffer put in both top and bottom chambers. Reservoir buffer was diluted from a ten times concentrated stock solution (0.25M Tris, 1.92M glycine, 1% SDS; pH8.3)

Prior to loading the sample, the gel was pre-run for 20 min at 80V (20mA) with the cathode as the upper (sample) reservoir.

The prepared samples (<100 μ l) were loaded into the sample slots, using a 0-100 μ l Hamilton syringe. Electrophoresis was performed at 80V until the sample dye had entered the resolving gel, and finally at 150V (initially 35mA) until the dye front reached the bottom of the gel (4-6h).

ii) Tris-Bicine method

A discontinuous buffer system, containing 0-1% SDS, was used. The final concentrations of buffers was: 0.1M Tris, 0.1M bicine in the stacking and resolving gels, and 0.02M Tris, 0.02M bicine in the reservoir buffer.

The resolving gel mixtures were made up according to table 3b for either 8-20%, or 8-25% gradients, and degased prior to the addition of TEMED. The resolving gel was poured exactly as for the 'Laemmli method', and overlayed with 1ml of water-saturated isobutanol. When set, the gel surface

Table 3 Recipes for gel preparation for Laemmli and Tris/bicine systems.

(a) Laemmli method

	<u>Resolving Gel</u>			<u>Stacking gel</u>
	8%	12%	20%	3.75%
Sucrose (\cong 2.7ml Vol.)	-	4.5g	4.5g	-
3M Tris HCl pH8.8	3.75ml	3.75ml	3.75ml	-
0.5M Tris HCl pH6.8	-	-	-	5.0ml
Acrylamide/bisacrylamide (30%/0.8%)	8.0ml	12.0ml	20.0ml	2.5ml
Water	17.05ml	10.55ml	2.55ml	11.3ml
1.5% ammonium persulphate	0.7ml	0.7ml	0.7ml	1.0ml
10% SDS	0.3ml	0.3ml	0.3ml	0.2ml
TEMED	10 μ l	10 μ l	10 μ l	15 μ l
Total Volume	30ml	30ml	30ml	20ml

Reservoir buffer: 0.025M Tris, 0.192M glycine, 0.1% SDS, pH8.3

Table 3

(b) Tris/Bicine method

	<u>Resolving Gel</u>			<u>Stacking Gel</u>
	8%	20%	25%	3.75%
Sucrose (\approx 2.7ml Vol.)	-	4.5g	4.5g	-
1M Tris, 1M Bicine	3.0ml	3.0ml	3.0ml	2.0ml
Acrylamide/bisacrylamide (47.6%/2.4%)	4.8ml	12.0ml	15.0ml	1.5ml
Water	21.2ml	11.3ml	8.3ml	15.3ml
1.5% ammonium persulphate	0.7ml	0.7ml	0.7ml	1.0ml
10% SDS	0.3ml	0.3ml	0.3ml	0.2ml
TEMED	10 μ l	10 μ l	10 μ l	15 μ l
Total Volume	30ml	30ml	30ml	20ml

Reservoir buffer: 0.02M Tris, 0.02M Bicine, pH8.2, 0.1M SDS.

was washed with water, which was subsequently removed. The stacking gel mixture was made up as in table 3b and degased prior to addition of TEMED. Following insertion of the comb into the glass plates, the stacking gel was loaded and allowed to set, after which the gel was treated as under the 'Laemmli method'. The pre-run was for 20 min at 80V (12mA). Electrophoresis of the sample was performed at 150V (20mA) through both the stacking gel and resolving gel, until the dye front reached the bottom of the gel (17-20h).

3.24 Sample preparation for SDS-Page

The procedure was identical for both the Laemmli and Tris-bicine methods. The sample buffer was prepared by dissolving 1.51g of Tris and 20ml glycerol in 35ml water, and adjusting to pH6.75 with concentrated hydrochloric acid. Following this, 4g SDS, 10ml 2-mercaptoethanol and 4mg bromophenol blue were dissolved in it, and the volume adjusted to 100ml.

Salts were removed from the samples by dialysis against 20mM Tris, pH8.0, or 125mM Tris, pH6.8. Samples which were freeze-dried were reconstituted with water. To the sample, in up to 50µl of Tris buffers, or water, was added an equal volume of sample buffer. The sample was boiled for 2-3 min, and spun in a microfuge at 17,000g for 5 min prior to loading the supernatant onto the

gel. Up to 100 μ l of sample was loaded per track (0.9mmdiam., 1.8mm depth).

3.25 Electrophoretic transfer of proteins from PAGE gels to nitrocellulose

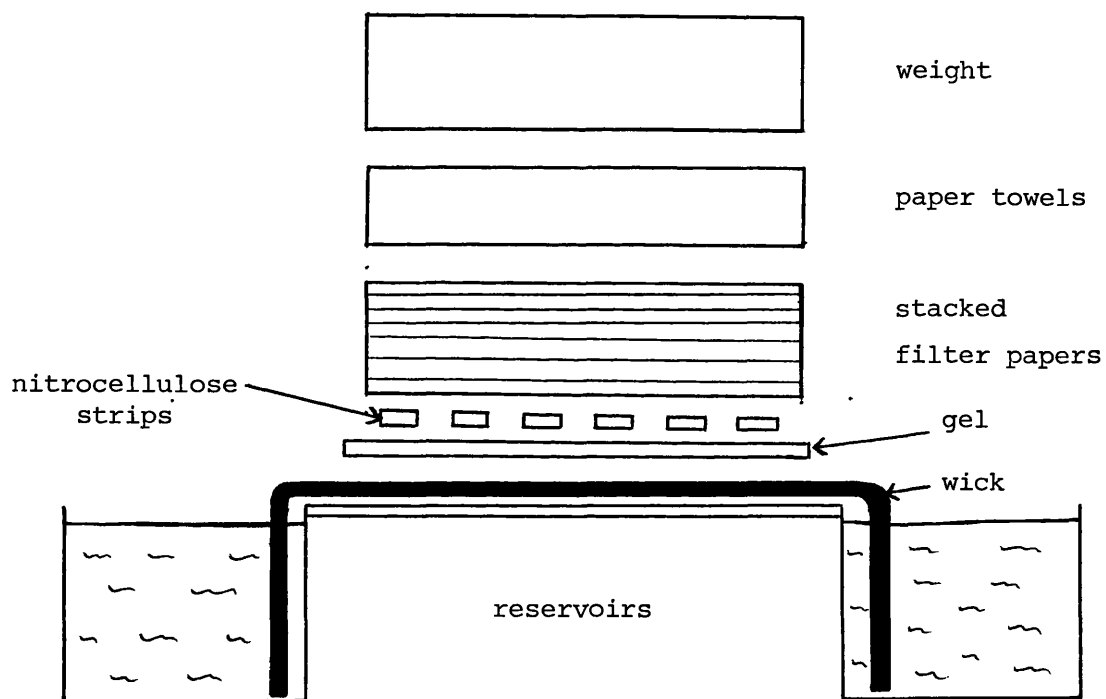
The blotting apparatus used was similar to the Bio-Rad Trans-BlotTM Cell, but had 2 graphite plates (175mm x 222mm x 10mm) as the electrodes.

After running as described in section 3.23, the resolving gel was removed carefully from the apparatus and tracks were marked with a scalpel. The gel was rinsed in distilled water and washed for 10 min in blotting buffer (2mM sodium acetate, 5mM MOPS, 20% ethanol, pH7.5). Nitrocellulose strips were cut to the same dimensions as the gel tracks, and washed for 10 min in the blotting buffer.

The gel was placed on a Bio-Rad Transblot sandwich pad, and the nitrocellulose strips were carefully placed over their corresponding tracks on the gel. The second sandwich pad was placed over this, and the apparatus was placed in the blotting tank, with the gel towards the cathode, and the nitrocellulose towards the anode. Electrophoretic transfer was carried out for 2h at 60V/0.55A, with stirring, and using a water cooling coil in the centre of the apparatus.

3.26 Passive transfer of proteins from PAGE gels to nitrocellulose

The apparatus used was set up as shown,below.



The buffer used in the reservoirs was 10mM Tris/HCl, pH 8.0, and transfer was performed for between 36h and 48h at room temperature.

3.27 Staining polyacrylamide gels

Tracks of gel to be stained for protein were incubated overnight at room temperature on a shaker, with 0.025% coomassie blue R stain in methanol/water/acetic acid (5:5:1). Destaining was achieved using several changes of 5% methanol, 10% acetic acid, 85% water.

3.28 Amido black staining of nitrocellulose bound protein

Nitrocellulose strips with blotted protein bands were incubated for 5 min with 1% naphthol blue-black in methanol/acetic acid/water (5:1:4). Destaining was performed in methanol/acetic acid/water (5:1:4).

3.29 Immunoblotting of nitrocellulose bound protein

Following electrophoretic transfer, nitrocellulose strips were incubated at 4°C with 1% casein in PBS overnight to block non-specific binding. Strips of 5mm width were fitted into 8-well multichannel pipette reservoirs for convenience for all further incubations.

Monoclonal antibodies were diluted to 100µg/ml, and sera used at a 1/50 or 1/100 dilution, in 1% casein/PBS. 4ml of antibody solution were used in each reservoir, and these were incubated for 2h on a shaker at room temperature. Strips were washed

for 5 x 10 min in 1% casein/PBS and incubated with goat anti-mouse immunoglobulins (polyvalent) for 2h at room temperature, with shaking. Strips were then washed for 5 x 10 min with PBS (azide free).

Colour was developed using: 0.02% 3-amino-9-ethyl carbazole, 5% dimethyl formamide, 0.00025% thiomersal, 0.03% H_2O_2 in 0.05M sodium acetate buffer, pH5.0, as substrate.

Fragmentation of PHCS

3.30 Limited proteolysis of PHCS

Proteolysis of PHCS by protease type VII, subtilisin BPN', chymotrypsin type II, from bovine pancreas and trypsin type III-S from bovine pancreas was performed at 25°C, as described by Bloxham et al, 1980 and Lill et al, 1984).

Prior to the reaction, PHCS was dialysed against 0.1M Tris-HCl, pH8.0. The final incubation mixtures contained PHCS (1mg/ml), a protease (subtilisin at 2µg/ml, chymotrypsin at 2µg/ml or trypsin at 10µg/ml), and palmitoyl CoA (250µM for chymotrypsin and subtilisin, 60µM for trypsin) in 50mM Tris-HCl, pH8.0. The reaction was stopped in all cases by the addition of two volumes of reaction mixture to 1 volume of 10mM phenylmethane-sulfonyl fluoride. Immediately after this, cold acetone (10 volumes) was added, and the digested PHCS fragments were precipitated by incubation at -20°C for 1h

followed by centrifugation in a microfuge at 17,000g.

The samples were reconstituted in water prior to the addition of PAGE sample buffer (section 3.24).

3.31 Chemical fragmentation of PHCS

Mild acid cleavage at aspartyl-prolyl peptide bonds was performed according to Rittenhouse and Marcus, 1984. PHCS (0.12mgml^{-1}) was incubated at 110°C in the presence of 15mM HCl. The reaction was stopped by rapid cooling and freeze-drying of the sample. The samples were reconstituted in water prior to the addition of PAGE sample buffer, section 3.24.

Hydroxylamine cleavage of asparaginyglycine bonds was performed according to Bornstein, 1970 and Bloxham et al, 1982.

Immediately prior to use, a reaction solution containing 1.8M hydroxylamine, 5.3M guanidine hydrochloride in 0.2M K_2CO_3 , pH10, was prepared as follows. $\text{NH}_2\text{OH}\cdot\text{HCl}$ (3.48g) and $\text{guanidine}\cdot\text{HCl}$ (14.32g) were dissolved in 13ml of H_2O in an ice bath. 2.5ml of 12.5N NaOH was added slowly with vigorous stirring, followed by addition of 5ml of 1M K_2CO_3 . The pH of the solution was titrated to 10.5 with additional NaOH, and the volume adjusted to 25ml.

For fragmentation to occur, 4 volumes of reaction solution were added to 1 volume of PHCS (1.60mg|ml), and the mixture was incubated at 45°C

for 7h. Following digestion, the sample was immediately dialysed at 0°C against 50mM Tris-HCl, pH8.0, 0.1% SDS, prior to the addition of PAGE sample buffer (section 3.24).

3.32 Preparation of mAb immunosorbent columns

The cyanogen bromide activation of Sepharose-4B, and subsequent coupling of mAbs, were performed according to March et al, 1974.

One volume of beads (Sepharose 4B), one volume of water, and two volumes of 2M sodium carbonate were mixed by stirring slowly. The rate of stirring was increased and 0.25 volumes of an acetonitrile solution of cyanogen bromide (0.9g cyanogen bromide per ml of acetonitrile) was added quickly. The slurry was stirred vigorously for 1.75 min, after which it was filtered over a coarse sintered-glass funnel and was washed with 10 volumes each of 0.1M sodium bicarbonate, pH9.5, water and 0.2M sodium bicarbonate, pH9.5. The filtered Sepharose was transferred to a plastic beaker containing one volume of 0.2M sodium bicarbonate, pH9.5, and one volume of mAb in 0.2M sodium bicarbonate, pH9.5 (3.3mg mAb per ml of Sepharose). Coupling was performed for 20h at 4°C. After coupling, the beads were washed with 0.1M sodium bicarbonate (pH10.0) and then incubated with 4 volumes of 1% casein in 0.2M sodium bicarbonate for 18h at 4°C. Beads were washed with 10 volumes each of 0.1M sodium acetate (pH4.0),

PBS and 0.1M sodium bicarbonate (pH10). The coupled beads were then packed into columns of approximately 1.5ml volume.

Before use, columns were washed with 10 volumes each of 1% casein in PBS, 0.5M NH_4OH , pH10.8, and PBS. Columns were stored and run at 4°C.

3.33 MAb immunosorbent chromatography

The sample to be loaded was reconstituted or equilibrated in PBS. For a column of 1.5ml volume, the sample (300µg protein in 1ml PBS) was loaded onto, and left to equilibrate in, the column for 6h. The columns were washed with 10 column volumes each of PBS, and 20mM Tris-HCl, pH8.0. The bound material was eluted with 2 column volumes of 0.5M NH_4OH , pH10.8, and freeze dried. The freeze dried eluants were reconstituted in water prior to the addition of PAGE sample buffer, section 3.24. Columns were re-equilibrated into PBS for storage.

Enzyme inhibition studies

3.34 Assay of citrate synthase activity

i) Acetyl CoA was prepared by the method of Stadtman, 1975. 10mg CoASH (free acid) was dissolved in 1ml of water, cooled to 0°C, and 0.2ml of 1M KHCO_3 was added to bring the pH to 7.5. 0.16ml of 0.1M acetic anhydride was added and the mixture was allowed to stand for 10 min. The acetylation was assumed to be complete if the acetyl CoA preparation was

unable to react with 10mM 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB).

ii) The enzymic assay for citrate synthase was performed as per Srere et al, 1963. All assays were carried out at 25°C. Unless otherwise stated the assay mixture contained (final concentrations) 0.15mM acetyl CoA, 0.2mM oxaloacetate and 0.1mM DTNB, in 20mM Tris-HCl, pH8.0/1mM EDTA. The reaction was monitored by measuring the increase in absorbance at 412nm due to the reaction of CoA with DTNB producing the yellow coloured thio-nitrobenzoate anion (E_{412} 13,600L.mol⁻¹.cm⁻¹ at pH8.0).

CHAPTER FOURProduction of mAbs

	Page
4.1 Comparison of immunization procedures	85
4.2 Production of mAb secreting cell lines A-E	85
4.3 Production of mAb secreting cell lines F, G, H, K, L and M	87
4.4 Production of mAbs from ascitic fluid	89
4.5 MAb sub-class typing	89
4.6 Further purification of mAbs	92
4.7 Purity of mAbs	93
4.8 Discussion	95

4.1 Comparison of immunization procedures

Four mice were given primary immunizations with PHCS, two intraperitoneally and two subcutaneously. After a period of 1 month, mice were boosted. Seven days later mice were tail bled, 20 μ l of blood being taken from each mouse. Sera obtained from the mice were assayed at dilutions of 1/100 by ELISA I. The results following a 10 min substrate incubation are shown in table 4.1.

These results show that intraperitoneal and subcutaneous primary immunizations were equally effective in inducing the production of antibodies against PHCS.

4.2 Production of mAb secreting cell lines A-E

One of the two subcutaneously primed mice, referred to above, was boosted to provide the spleen for fusion. The fusion was performed as in section 3.8, and the cells were plated out onto 5 microtiter plates.

446 of the 480 possible wells showed colony growth, equivalent to 93% of wells. The fraction of wells with growth follows the Poisson distribution (Goding, 1980).

Using this theorem $f(0) = e^{-\lambda}$, where $f(0)$ is the fraction of wells with no growth and λ is the average number of clones per well. It is estimated that on average each well of the fusion contained 2.65 growing colonies.

Table 4.1 Anti-PHCS activity shown by sera from mice primed intraperitoneally and subcutaneously

<u>Serum</u>	<u>Average</u>
	A_{405}
1P 1	1.28
1P 2	1.40
SC 1	1.46
SC 2	1.35
Norm. Mouse Ser.	0.04
PBS	0.02

Anti-PHCS activity, measured by ELISA I, is expressed as the A_{405} value reached following a 10 min substrate incubation.

Using ELISA I, all wells showing growth were assayed for anti-PHCS activity. Supernatants from 31 wells were strongly positive ($\text{Abs}_{405} > 0.3$ after 30 min incubation with substrate) and 25 wells were weakly positive ($\text{Abs}_{405} > 0.15$). This suggests that 4.4% of all growing colonies were secreting antibodies reactive with PHCS.

5 cell lines A-E were produced, cloned and grown up in mice, from which ascitic fluid was collected. During cloning, supernatants were assayed by ELISA I. The cloning efficiencies are shown in table 4.2.

4.3 Production of mAb secreting cell lines F, G, H, K, L and M

A mouse was primed intraperitoneally, boosted 7 days later and tail bled (20 μ l blood) 7 days after this. The serum obtained was assayed by ELISA VIII and proved to be positive for anti-PHCS when compared to normal mouse serum. Two days after tail bleeding, the mouse was boosted and 4 days following this its spleen cells were used in a fusion. The fusion was performed as in section 3.8 and was plated out onto 5 microtiter plates.

Supernatants of two plates were screened for the ability to inhibit enzyme activity, when incubated for 15 min with 6ng of PHCS. The reaction was performed in ELISA plates and started by addition of acetyl CoA and oxaloacetate (to final concentrations of 10 μ M). Following a 20 min incubation, plates were

Table 4.2 Cloning efficiencies of the 5 cell lines A-E

Cell Line	<u>Once cloned</u>		<u>Twice cloned</u>		<u>Three times cloned</u>		<u>Four times cloned</u>	
	cell growth	anti-PHCS	cell growth	anti-PHCS	cell growth	anti-PHCS	cell growth	anti-PHCS
	% +ve	% +ve	% +ve	% +ve	% +ve	% +ve	% +ve	% +ve
A	$\frac{33}{80}$ =41%	$\frac{21}{33}$ =64%	$\frac{22}{80}$ =28%	$\frac{17}{22}$ =77%	$\frac{42}{80}$ =53%	$\frac{36}{42}$ =86%	N/A	N/A
B	$\frac{24}{114}$ =21%	$\frac{2}{24}$ =8%	$\frac{21}{80}$ =26%	$\frac{9}{21}$ =43%	$\frac{29}{80}$ =36%	$\frac{26}{29}$ =89%	N/A	N/A
C	$\frac{47}{80}$ =59%	$\frac{26}{47}$ =55%	$\frac{48}{80}$ =60%	$\frac{26}{48}$ =54%	$\frac{34}{80}$ =43%	$\frac{24}{34}$ =71%	$\frac{21}{80}$ =26%	$\frac{15}{21}$ =71%
D	$\frac{31}{80}$ =39%	$\frac{3}{31}$ =10%	$\frac{28}{80}$ =35%	$\frac{12}{28}$ =43%	$\frac{32}{80}$ =40%	$\frac{32}{32}$ =100%	N/A	N/A
E	$\frac{8}{80}$ =10%	$\frac{7}{8}$ =88%	$\frac{21}{80}$ =26%	$\frac{20}{21}$ =95%	$\frac{18}{80}$ =23%	$\frac{15}{18}$ =83%	N/A	N/A

Cell growth is expressed as the percentage of seeded wells which grow colonies. The percentage of wells showing colony growth together with anti-PHCS activity is also shown.

read at 405nm on an ELISA reader. Two cell lines (clones L and M) appeared to be secreting inhibitory antibodies. Following incubation with supernatants from these cell lines, PHCS gave a rate of reaction of $0.02\Delta A_{405}/\text{min}$ as compared with a rate of $0.027\Delta A_{405}/\text{min}$ following incubation with antibody free supernatants, and a rate of $0.002\Delta A_{405}/\text{min}$ for no-enzyme controls.

The three further plates were screened using ELISA VIII, taking an A_{405} of >0.150 after 30 min incubation with substrate as positive. By this screen a further 4 cell lines were chosen, F, G, H and K.

All six cell lines from this fusion were screened by ELISA VIII during cloning. The cloning efficiencies are shown in table 4.3.

4.4 Production of monoclonal antibodies from ascitic fluid

In subsequent chapters, the mAbs are named after the cell line from which they are produced.

Table 4.4 shows the number of mice from which ascitic fluid was collected for each cell line, and the quantity of mAbs obtained following ammonium sulphate precipitation.

4.5 MAb sub-class typing

Partially purified mAbs were used at a concentration of $5\mu\text{g/ml}$, and the assay was performed as described in section 3.22.

Table 4.3 Cloning efficiencies of cell lines F, G, H, K, L and M

Cell line	<u>Once cloned</u>		<u>Twice cloned</u>	
	cell growth % +ve	anti-PHCS % +ve	cell growth % +ve	anti-PHCS % +ve
F	$\frac{34}{80}$ =43%	$\frac{24}{34}$ =71%	$\frac{9}{80}$ =11%	$\frac{4}{9}$ =44%
M	$\frac{15}{80}$ =19%	$\frac{6}{15}$ =40%	$\frac{23}{80}$ =29%	$\frac{16}{23}$ =70%
G	$\frac{40}{80}$ =50%	$\frac{15}{40}$ =38%	$\frac{19}{80}$ =24%	$\frac{9}{19}$ =47%
H	$\frac{27}{80}$ =34%	$\frac{12}{27}$ =44%	$\frac{17}{80}$ =21%	$\frac{8}{17}$ =47%
K	$\frac{47}{80}$ =59%	$\frac{35}{47}$ =74%	$\frac{42}{80}$ =53%	$\frac{38}{42}$ =90%
L	$\frac{38}{80}$ =48%	$\frac{30}{38}$ =79%	$\frac{51}{80}$ =64%	$\frac{32}{51}$ =63%

Cell growth is expressed as the percentage of seeded wells which grew colonies. The percentage of wells showing colony growth together with anti-PHCS activity is also shown.

Table 4.4 Quantities of mAbs obtained from ascites

mAb name	no. mice used	Protein after Amm. Sulphate preciptn. (mg)
A	8	650
B	5	370
C	8	700
D	8	90
E	8	150
F	5	50
G	5	180
H	5	190
K	5	160
L	5	50
M	5	30

Two mAbs, B and C, showed only a single precipitin line between the mAb well and the well containing sheep anti-mouse IgG1. 8 mAbs (A, D, E, F, G, H, K, L and M) were similarly shown to be IgM.

4.6 Further purification of mAbs

Further purification of mAbs A-E was attempted using insolubilized Protein A, by the method described in section 3.16, while monitoring the fractions collected (following neutralization by dropwise addition of 2M Tris base) by ELISA I.

MAbs A, D and E are of IgM class and did not bind to the insolubilized Protein A. MAb C, an IgG1, bound only slightly and it was not considered worthwhile trying to purify it further by this method.

MAb B, an IgG1, was purified using immobilized Protein A. 98mg of partially purified mAb B, in 4.5ml of 0.1M sodium phosphate buffer, pH8.0, was loaded onto a 15ml Protein A affinity column. Washing and elution was performed as in 3.16. Substantial anti-PHCS activity was found in the 0.1M sodium citrate pH6.0 fraction, which contained 72.5mg protein. No anti-PHCS activity was detected in the 0.1M phosphate washing buffer, the citrate eluting buffers pH4.5, pH3.5, or in the final 0.1M citric acid wash. The combined protein eluted by other washing and eluting buffers was \approx 40mg. This indicates that only a 1.5 fold purification was achieved using Protein A affinity chromatography and therefore this method was not adopted routinely.

4.7 Purity of monoclonal antibodies

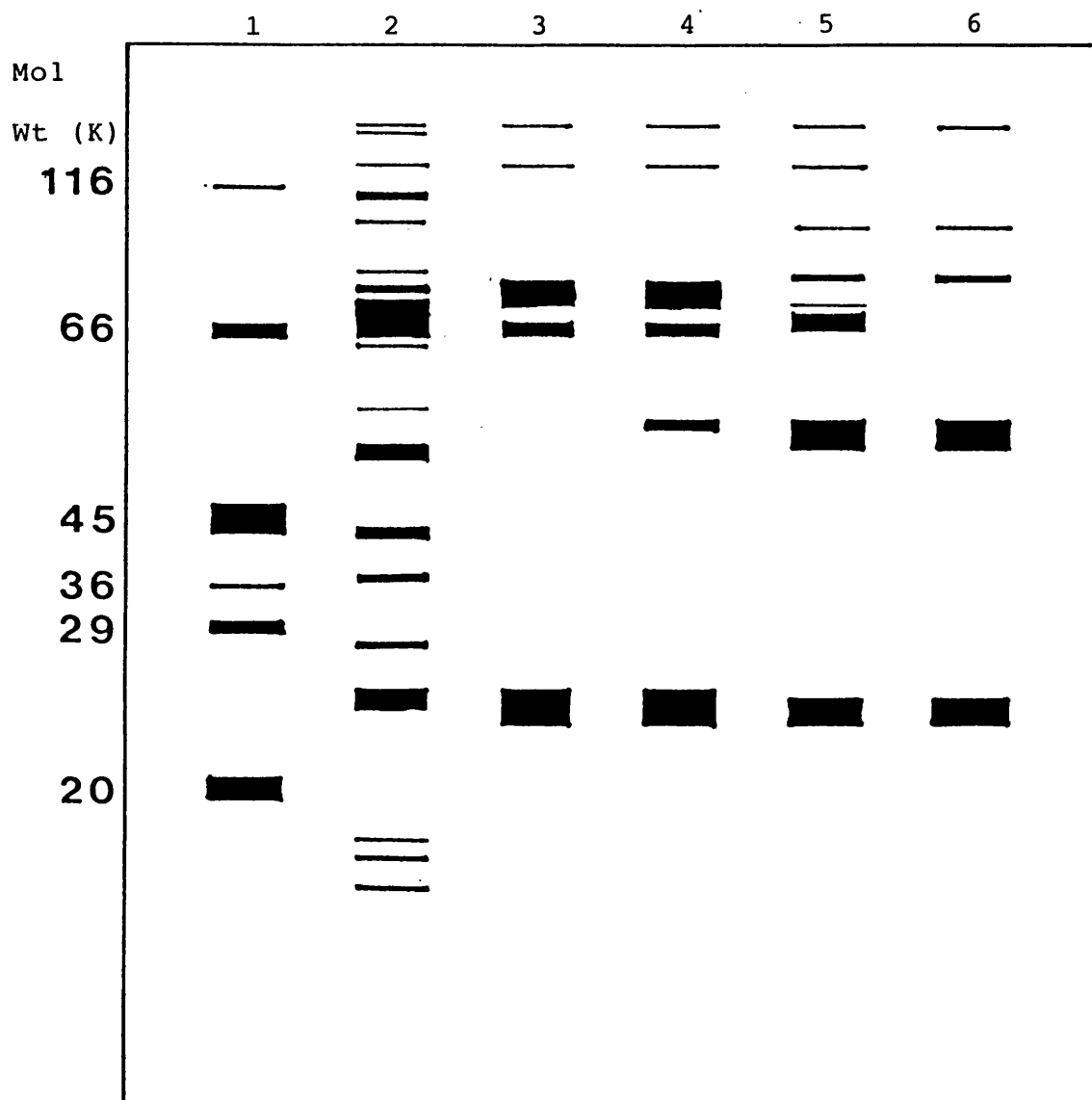
The purity of mAbs was assessed by SDS PAGE under reducing conditions (section 3.23i). An 8-12% acrylamide gradient gel was used. Normal mouse serum was included on the gel together with standards (fig 4.1).

MAb B, an IgG1 purified on immobilized Protein A, showed bands corresponding to light chains (25K), γ chains (52K) and 2 feint bands at 75K and 100K (track 6). MAb C, an IgG1 partially purified using ammonium sulphate, showed these bands plus a band corresponding to human albumin (68K) and a feint band at 70K (track 5).

Track 3 is a sample representing all of mAbs D, E, F, G, H, L and M, which all show bands at 25K (light chains), 67K (albumin) and 71-73K (μ chain). MAb A shows these bands plus a band at 53K.

All mAb tracks showed a substantial increase in purity over the normal serum track. The major non-immunoglobulin contaminant being albumin, which by the intensity of its staining was estimated as comprising less than 10% of the total protein on tracks 3-5.

Fig 4.1 Schematic representation of an 8-12% PAGE gel to show the purity of mAbs



Track 1 molecular weight markers; B-galactosidase (116K), bovine serum albumin (66K), ovalbumin (45K), glyceraldehyde 3-phosphate dehydrogenase (36K), carbonic anhydrase (29K) and bovine trypsin inhibitor (20K).

Track 2 normal mouse serum, track 3 mAbs D, E, F, G, M, L or M, track 4 mAb A, track 5 mAb C, track 6. mAb B.

4.8 Discussion

The fusion partner X63-Ag8.655 was chosen as it has entirely lost immunoglobulin expression, and therefore does not secrete immunoglobulin heavy or light chains. The generation of hybridomas was performed by the method of Fazekas de St. Groth and Scheidegger (1980), who have optimized the techniques presently used to produce mouse monoclonal antibodies.

Most cell fusions are now performed with PEG (Zola and Brooks, 1982). The use of DMSO however, is not so well accepted as being necessary. Fazekas de St. Groth and Scheidegger (1980) reported no improvement in the number of hybridomas on addition of DMSO; however the reverse was found by Westerwoudt et al, 1984. We included 5% DMSO as a harmless precaution in the fusion mixtures.

Both PEG and DMSO markedly decrease the surface potential of biological membranes (Maggio et al, 1976) and cause charge neutralization of the cell membrane. Dehydration may also have a role in PEG-mediated fusion (Knutton and Pasternak, 1979).

With an electron microscope small cytoplasmic bridges can be observed between cells about 4 min after exposure to PEG (Knutton, 1979). This intercytoplasmic communication begins 1-3 min after dilution of PEG (Wojcieszyn et al, 1981). Water-soluble cytoplasmic proteins do not diffuse from one cell to another until PEG is removed (Westerwoudt, 1985). Cytoplasmic mixing is optimal at 37°C and is complete

at 4h (Wojcieszyn et al, 1983). At this stage a heterokaryon exists (with two or more nuclei). During the next cell division the nuclei fuse and a hybrid cell results (Goding, 1980).

Following HAT selection, a fusion mixture would contain cells secreting mAbs against PHCS, cells secreting mAbs against irrelevant antigens and cells secreting no antibody. It is necessary to clone cells as soon as they have been screened for antibody production in order to separate out the mAb secreting cells required.

The method of cloning cells by limiting dilution does not in practice always generate Abs that are truly monoclonal (Galfre and Milstein, 1981). In this study we used a single cell transfer method which should produce monoclonality following each cloning step (Gagnon and Raymond, 1985).

Immediately following hybridisation, the hybrid cells will have approximately 80 chromosomes and as they divide they may randomly lose some of these chromosomes. Plasma cells may devote up to half their total protein synthesis to that of immunoglobulin (Goding, 1980). A cell which sheds this burden, by loss of chromosomes, will in all likelihood grow faster. Thus there will be a strong tendency for cultures to be overgrown by non-producing cells, emphasizing the need for early cloning.

From my results of the number of growing wells positive for anti-PHCS activity at each stage of cloning, an increase is seen with increased cloning.

Summing these results for the 11 hybridomas it is seen that following the first cloning 181 of 344 cells were secreting anti-PHCS Abs (53%). This percentage is a reflection of the fact that a positive antibody secreting well after fusion may contain colonies derived from more than one fused cell (c.f. 2.65 colonies/well for first fusion). These will be carried through at the first cloning. Following the first cloning by single cell transfer, each well now contains a colony derived from one single cell. Subsequent clonings from positive anti-PHCS secreting wells might be expected to yield only anti-PHCS positive colonies. However my results (summed for 11 hybridomas) indicate that after two clonings only 191 of 301 colonies have anti-PHCS activity (63%), and after three clonings 133 of 155 colonies (86%) have anti-PHCS activity.

These results show that some cells derived from secreting parent cells lose their ability to secrete immunoglobulins. This may be by chromosome loss as previously discussed. The increase in the percentage of growing colonies secreting anti-PHCS activity seen with increasing cloning does however show that this technique is very useful in strengthening a hybridoma cell line.

As soon as feasible, aliquots of cells were preserved in liquid nitrogen. This was to provide backup cell stocks at each stage of cloning to ensure against loss of cells by overgrowth of non-producers

or by infections.

Macrophages(peritoneal cells) were used as feeder cells at all stages when cells were transferred from one culture vessel to another, or when they were to be grown at low density (ie. upon cloning). Macrophages are able to clear dead cell debris from cultures and probably also secrete 'growth factors' which may stimulate the growth of hybridomas. Where growth of hybridomas is at low density, use of macrophages creates an important increase in overall cell concentration and may also remove toxic by-products from the medium (Zola and Brooks, 1982).

The ratio of IgG to IgM secreting hybridomas may be dependent on the method of immunisation. In this study an initial subcutaneous immunisation followed by two boosters at weeks 5 and 6 gave 2 IgG1 secreting hybridomas and 3 IgM secreting hybridomas. The subsequent fusion involving intraperitoneal priming and two booster injections at weeks 2 and 3 led to 6 IgM secreting hybridomas. It has been suggested that increased time of immunisation, multiple boosters and even intra-splenic immunisation, may be used to increase the proportion of IgG secreting hybridomas obtained.

The assessment of the purity of the mAbs (following ammonium sulphate purification) shows the major non-immunoglobulin contaminant to be albumin. The content of specific mAb, within the immunoglobulin fraction of ascitic fluid purified by ammonium sulphate precipitation, has not been assessed. It

is however generally accepted that ascitic fluid containing mAbs is highly specific and can be used directly in immunochemical studies (Goding, 1980, Mierendart, 1983, Friguet, 1983).

Using the different screening methods described, this work has generated a library of 11 mAbs. The library of mAbs, to be used further in studying antigenicity, is required to have a large range of different specificities. Various screening methods were used as the use of different screening methods enables selection of mAbs with a wide range of specificities, discussed further in chapter 5.

CHAPTER FIVE Immunochemical screening methods

	Page
5.1 Choice of optimum conditions for ELISAs and immunodot blots	101
5.2 Hybrid screening using ELISAs IV and VI	104
5.3 Comparison of solid phase immunoassays	104
5.4 'Open' and 'Closed' forms of PHCS used in sandwich ELISAs	123
5.5 The effect of SDS and 2-mercaptoethanol on the presentation of PHCS in sandwich ELISAs	127
5.6 Discussion	130

5.1 Choice of optimum conditions for ELISAs and immunodot blots

On an ELISA plate it is convenient to vary up to two parameters simultaneously, one in either direction, to determine the optimum combination of the two for future use. This is called a checkerboard system.

Using a series of dilutions of mouse anti-PHCS serum and varying the concentration of coating antigen in assays I-VII, the conditions for antigen coating were optimized. For the sandwich assay (VIII) both the concentration of coating rabbit IgG anti-PHCS and the following antigen, were varied for optimization.

Figures 5.1 and 5.2 show results for the optimization of assays IV and V as examples. From these results optimal sensitivity was achieved by using PHCS at 10 or 20 μgml^{-1} for both assays. It was decided to use PHCS routinely with poly L-aspartate pre-coated plates at 10 μgml^{-1} in carbonate buffer, for assay IV, and at 10 μgml^{-1} in PBS for assay V. Similar experiments for the other assays led to the optimum coating conditions given in section 3.18 and 3.20.

The final substrate concentration (1 mgml^{-1} p-nitrophenyl phosphate) was chosen, as it provides the substrate in excess, such that a linear rate of change in absorbance is given up to a value of 2 absorbance units at 405nm.

Fig 5.1 The effect of varying the concentration
of PHCS on the sensitivity of ELISA IV

Substrate incubation was for 30 min

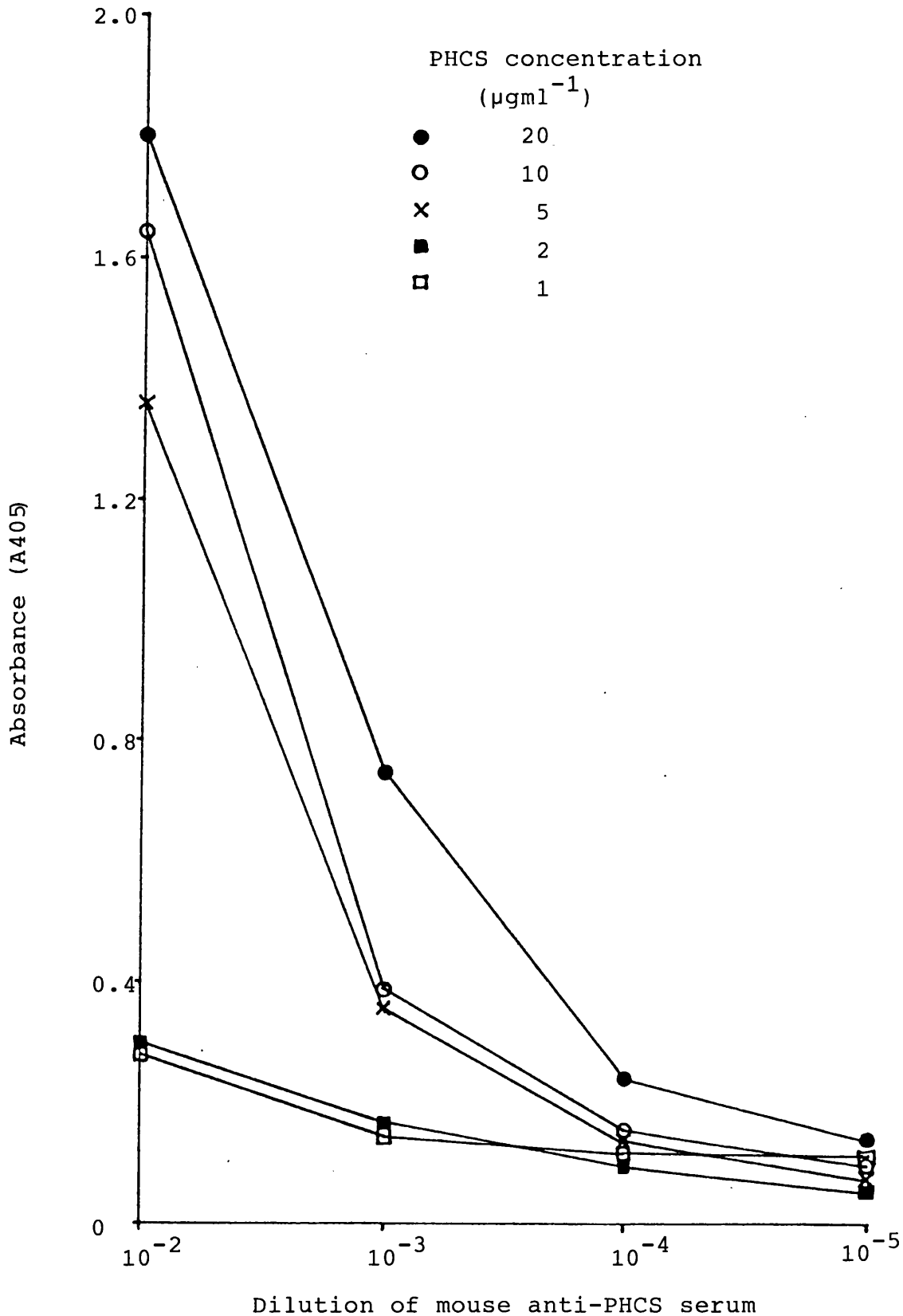
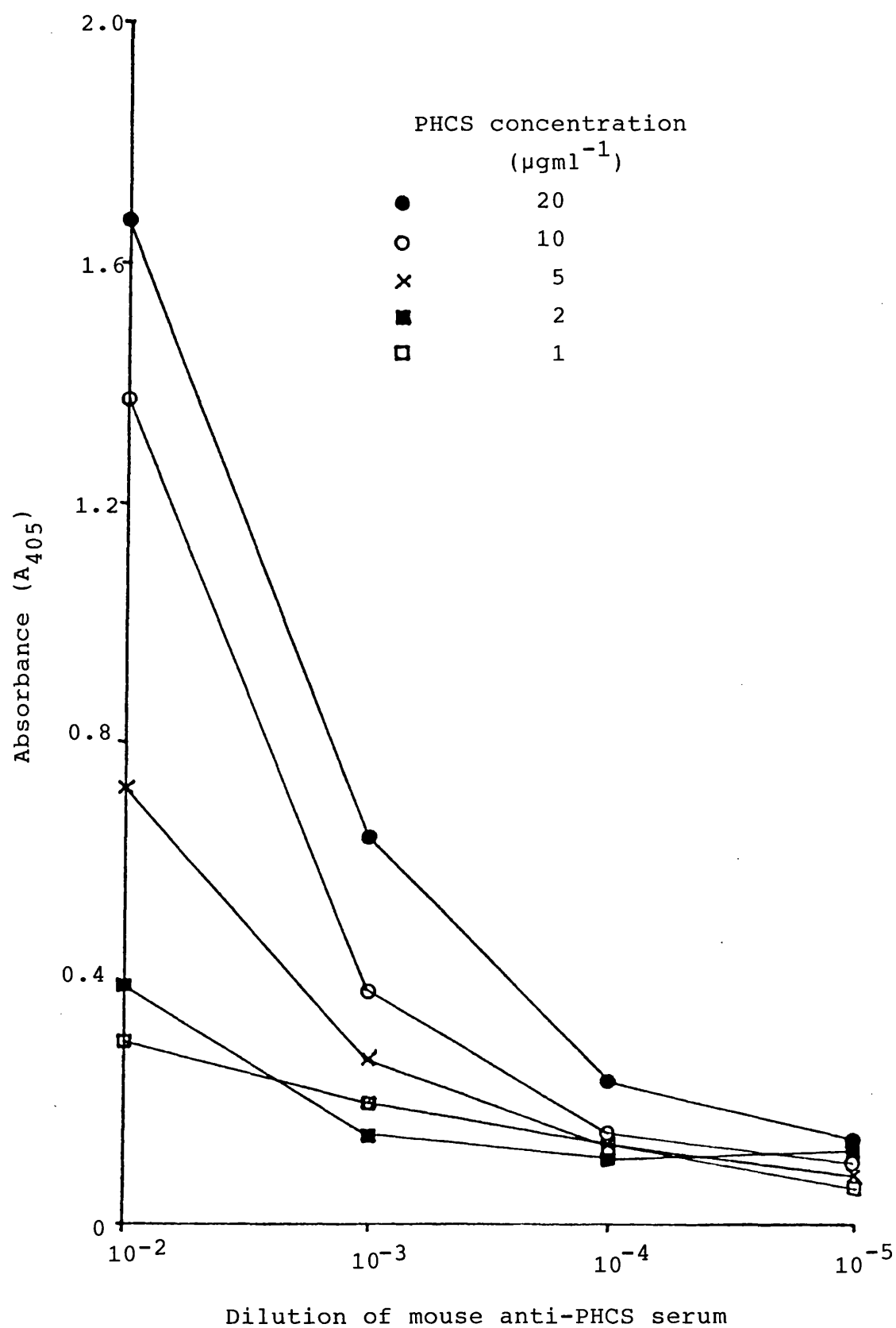


Fig 5.2 The effect of varying the concentration of PHCS on the sensitivity of ELISA V

Substrate incubation was for 30 min.



5.2 Hybrid screening using ELISAs IV and VI

ELISA systems with poly L-aspartate or poly L-lysine spacers (assays IV and VI) were compared as alternative initial screens for anti-PHCS producing hybridomas. Following cell fusion, a 96 well culture plate containing 2×10^6 myeloma cells and 2×10^7 immunized spleen cells in HAT medium was incubated for three weeks, at which stage supernatants were harvested and cultures fed with fresh medium. Supernatant harvesting was continued at two day intervals and samples were stored at -20°C prior to assay.

Pooled samples from each of the 56 colony-containing wells were assayed for anti-citrate synthase using assays IV and VI. An average absorbance (of duplicate assays) at 405nm of greater than 0.09 units following a 30 min incubation with substrate, was considered positive. The results (table 5.1) show that although certain colonies were identified as anti-PHCS secretors by both assays, other colonies were identified by only one of the assays. Thus of the 14/56 colonies which produced anti-PHCS, 4/14 were identified only by assay IV, 4/14 only by assay VI and 6/14 by both assays. This suggests that neither of the assays used for screening is able to detect all antibody-producing cultures.

5.3 Comparison of solid phase immunoassays

The relative reactivities of the mAbs were compared using the various antigen coating conditions

Table 5.1

well no.	anti-PHCS	
	screen	
	assay IV	assay VI
A10	-	+
D1	-	+
B5	+	+
A1	+	+
G10	-	+
A3	+	+
G3	+	+
A9	-	+
D12	+	+
F4	+	+
C9	+	-
F2	+	-
D11	+	-
G6	+	-

Anti-PHCS activity in culture supernatants was measured using assay IV or assay VI. Positive reaction was defined as an increase in A_{405} of >0.09 after incubation with substrate for 30 min.

for ELISAs and immunodot blots given in section 3.18 and 3.20. MAbs were used at concentrations of 100, 10, 1 and $0.1\mu\text{gml}^{-1}$. The results were expressed as graphs of A_{405} versus concentration of antibody for assays I-XII (figs 5.3-5.14). The antibody activity for each mAb (at $100\mu\text{gml}^{-1}$ concentration) was expressed as a percentage of the A_{405} value given by a 1/50 dilution of mouse serum anti-PHCS. The data are presented in the form of histograms (figs 5.15-5.18).

From these data it can be seen that when direct coating assays (I-III) were compared, where the pH of the antigen-coating buffer was varied, little difference was seen in the relative reactivity of each mAb between the different assays (figs 5.3, 5.4, 5.5 and 5.15). Similar relative reactivities were also observed when comparing the use of poly-L-aspartate as spacer (figs 5.6, 5.7 and 5.16) with direct coating (fig 5.15). In all these assays, mAbs B, C, E and F show strong reactivity whereas A, H and L appear relatively weak. In procedures VI and VII, where poly-L-lysine was used as spacer, mAbs F and E gave a strong reaction, as did B and C in assay VI (figs 5.8, 5.9 and 5.16). MAbs H and L, which were very weak responders in assays I-V, gave a stronger response in assay VI and VII.

The sandwich ELISA (assay VII), in which antigen was immobilized via polyclonal rabbit anti-PHCS, showed increased reactivity with all mAbs (figs 5.10 and 5.17) when compared with direct assays. Clones B, D, E, F, H and K all showed > 50% of the reactivity

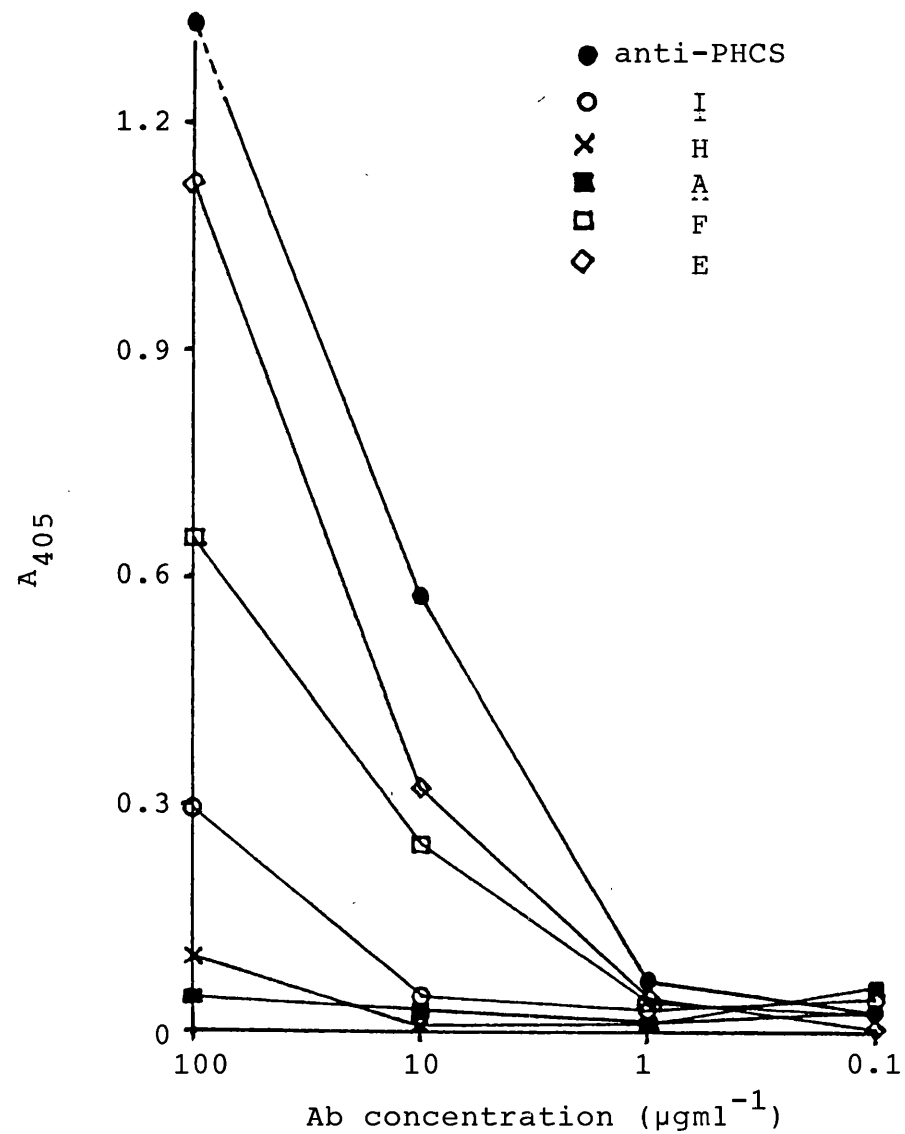
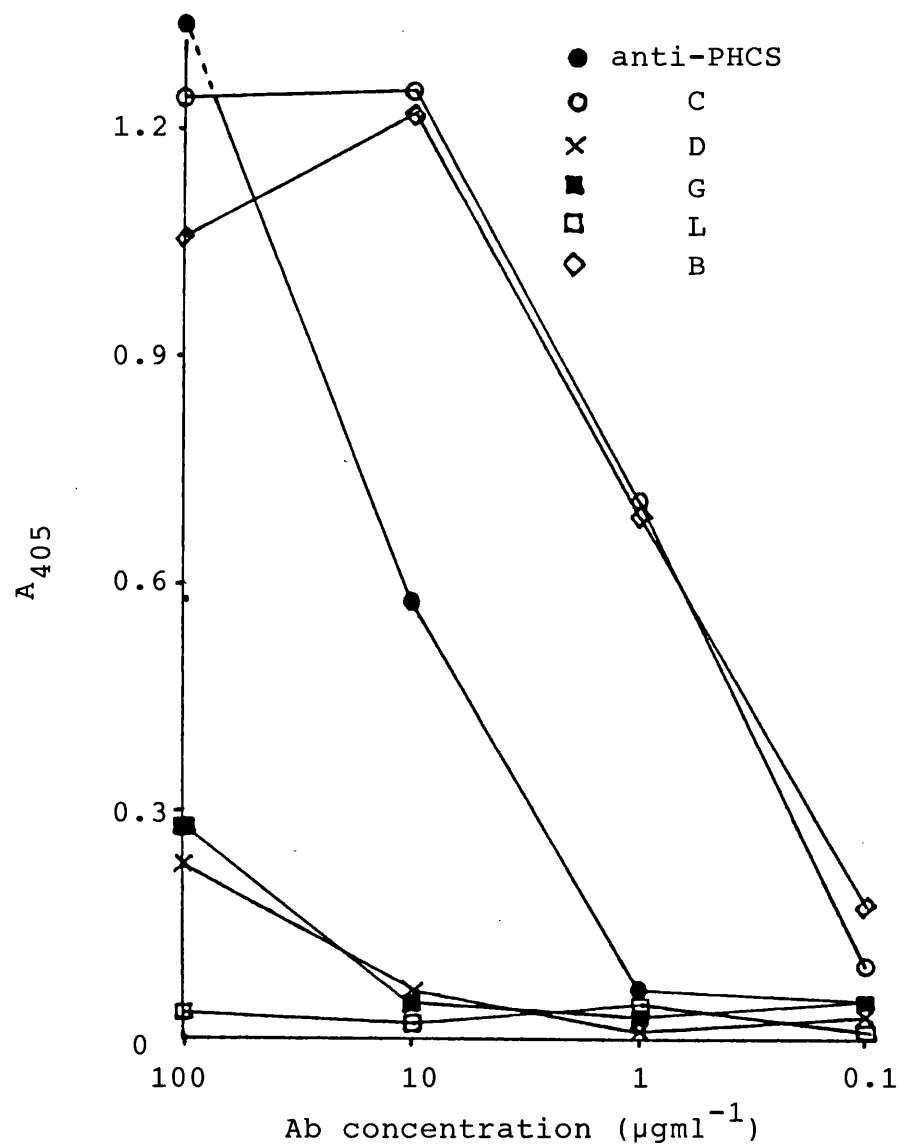


Fig 5.3 Dilution curves for mAbs in ELISA I (PHCS coated directly at pH9.8). Mouse anti-PHCS serum ● was used at dilutions of 1/50, 1/500, 1/5x10³, and 1/5x10⁴. Substrate incubation was for 30 min.

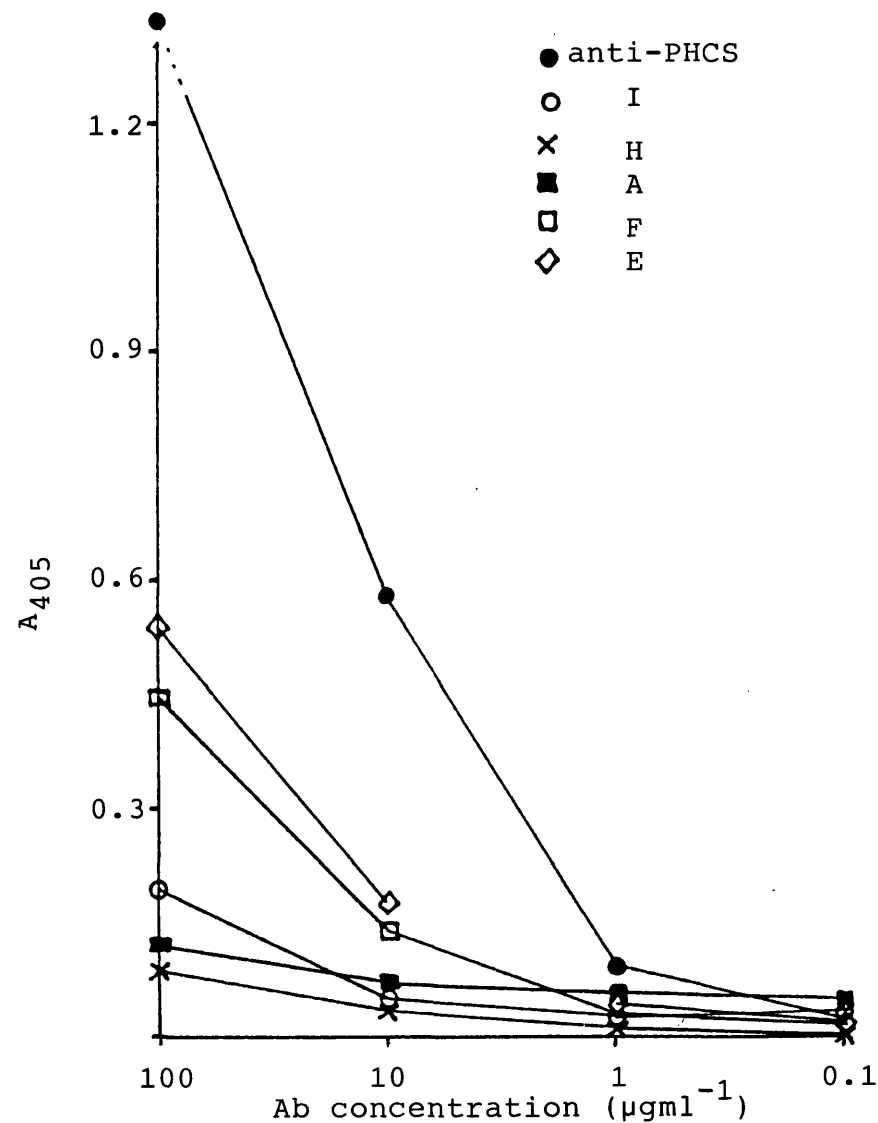
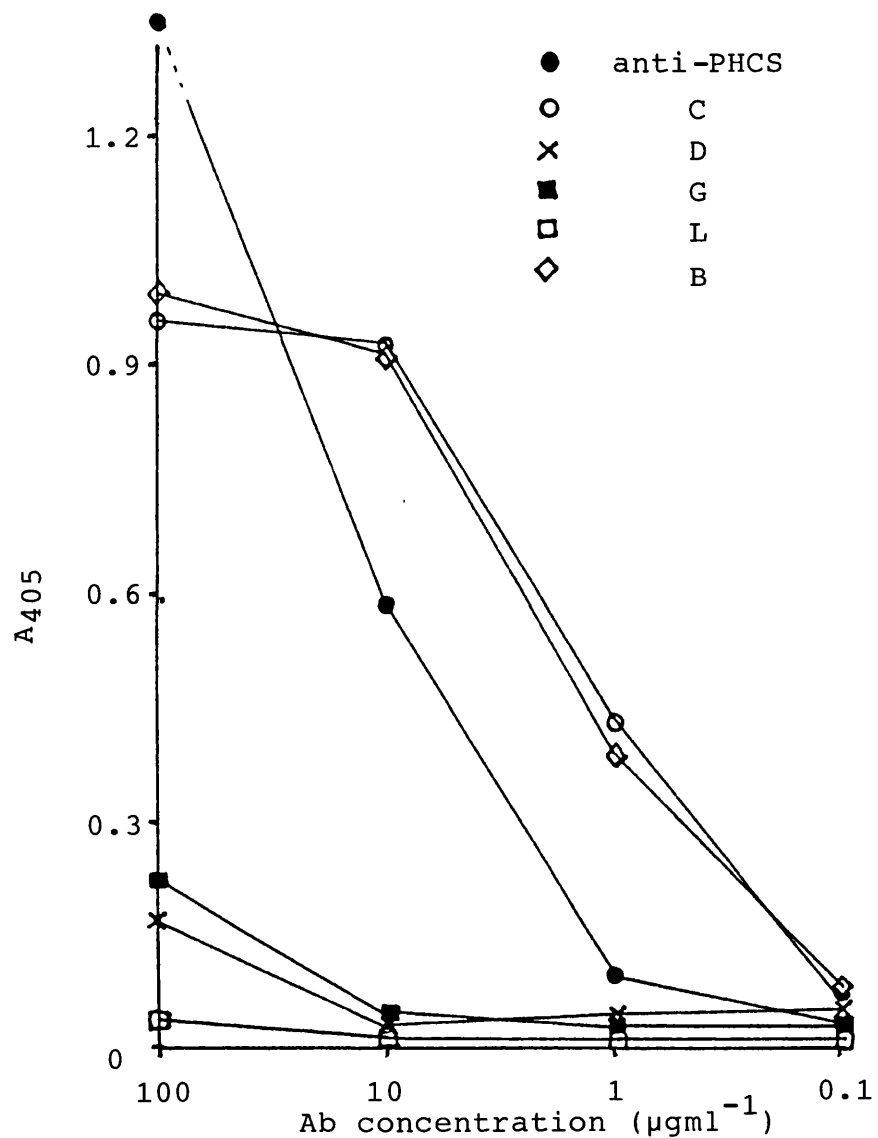


Fig 5.4 Dilution curves for mAbs in ELISA II (PHCS coated directly at pH7.3)

Mouse anti-PHCS ● was used at dilutions of 1/50, 1/500, 1/5x10³ and 1/5x10⁴.

Substrate incubation was for 30 min.

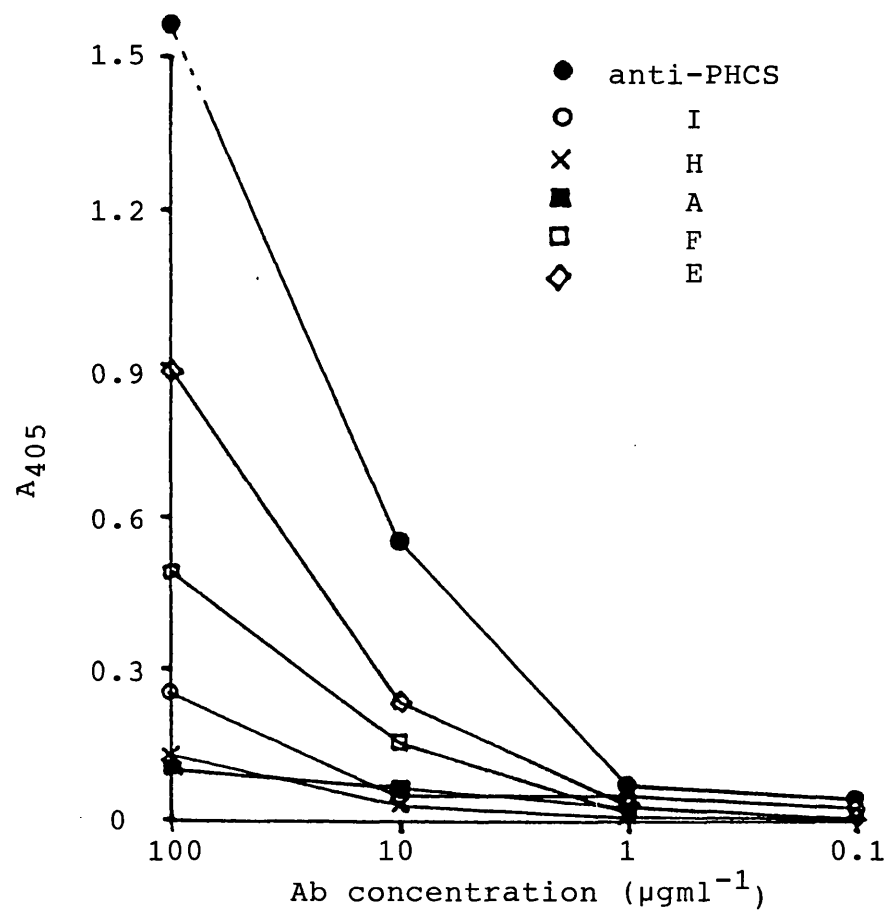
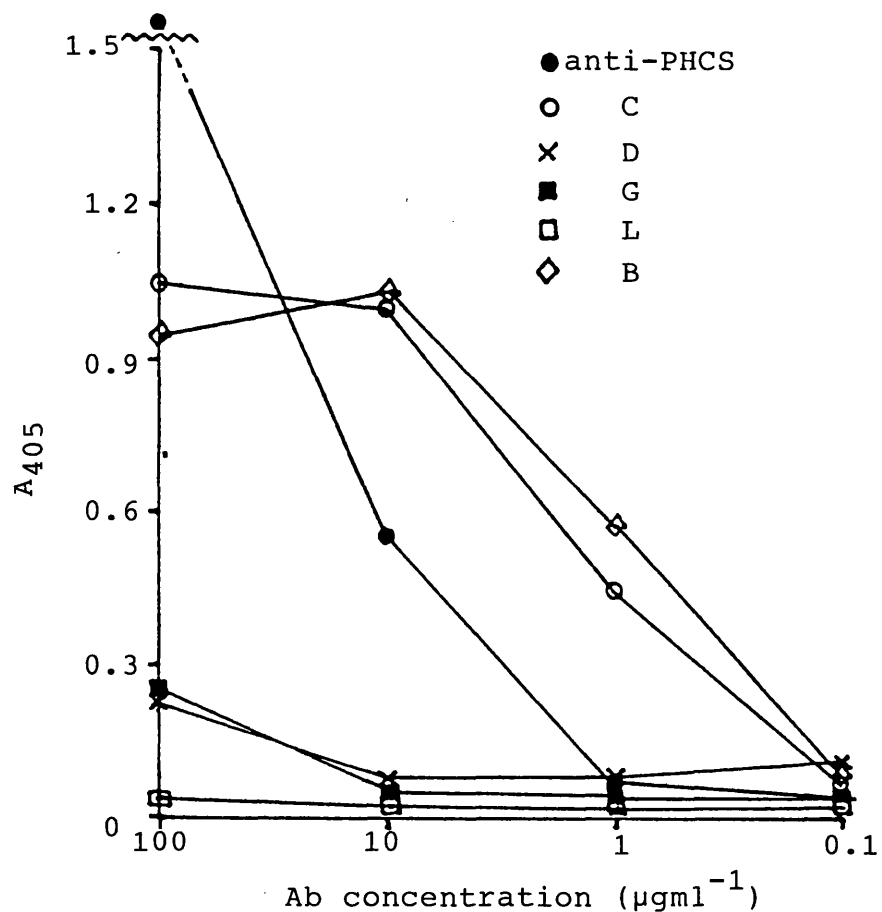


Fig 5.5 Dilution curves for mAbs in ELISA III (PHCS coated directly at pH4.2)
 Mouse anti-PHCS serum ● was used at dilutions of 1/50, 1/500, 1/5x10³ and 1/5x10⁴
 Substrate incubation was for 30 min.

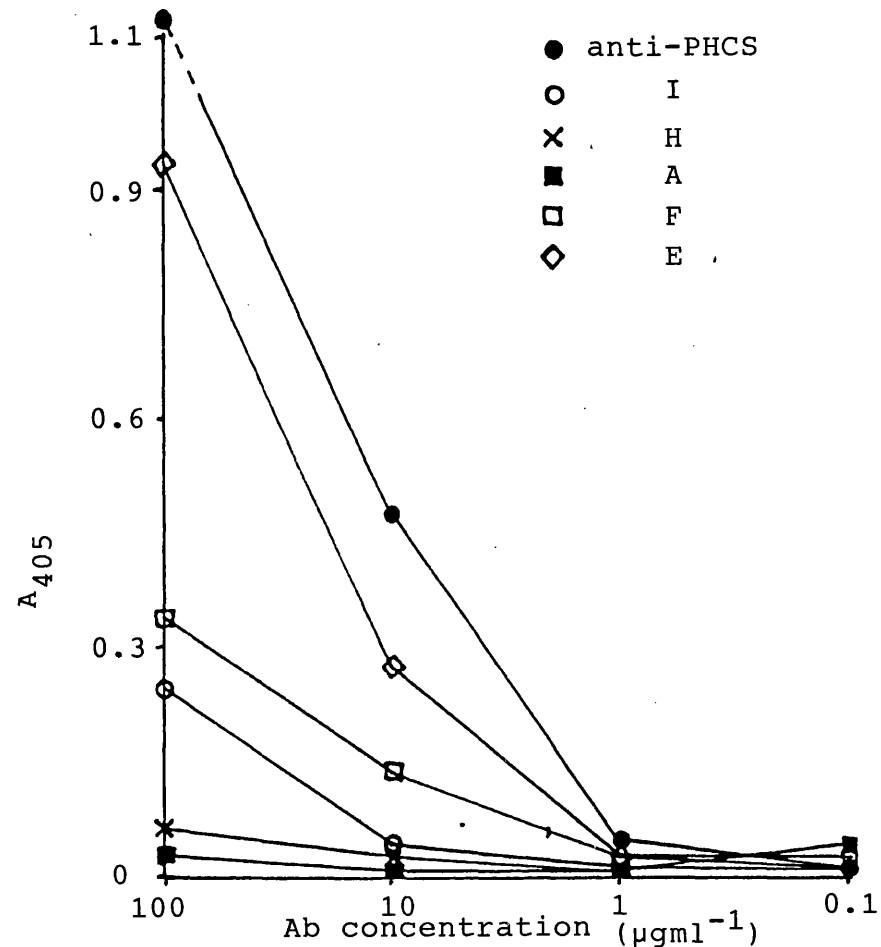
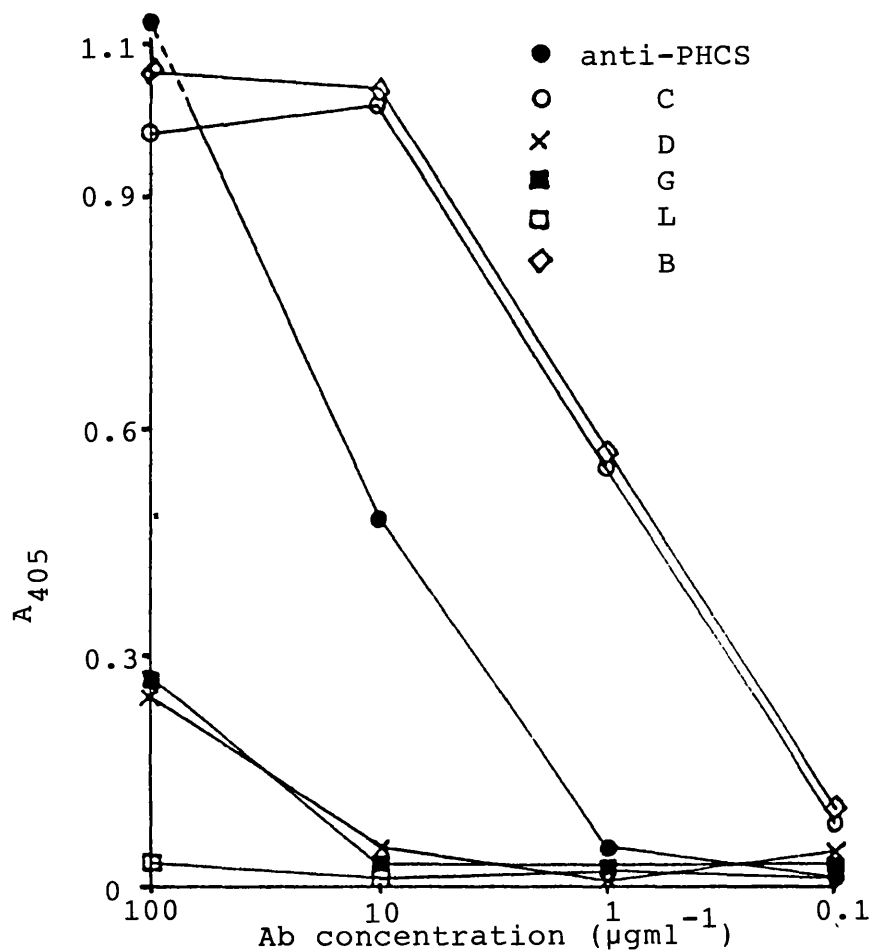


Fig 5.6 Dilution curves for mAbs in ELISA IV (poly-L-aspartate pre-coated plate coated with PHCS at pH 9.8). Mouse anti-PHCS serum ● was used at dilutions of 1/50, 1/500, 1/5x10³ and 1/5x10⁴. Substrate incubation was for 30 min.

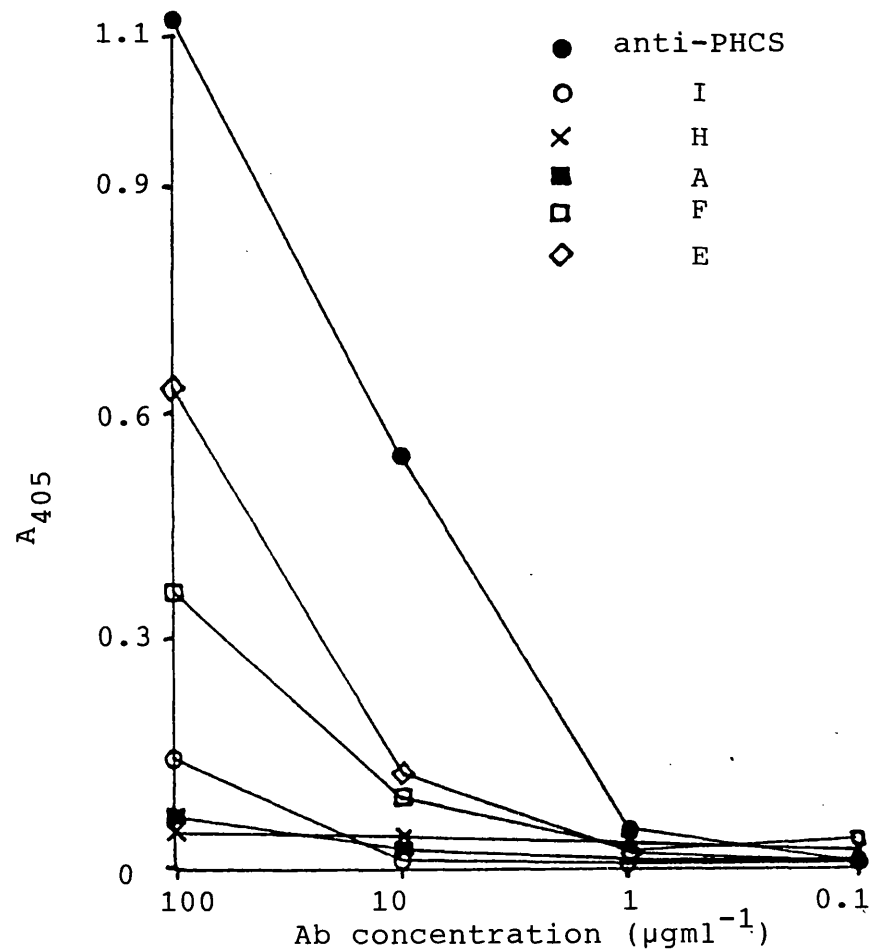
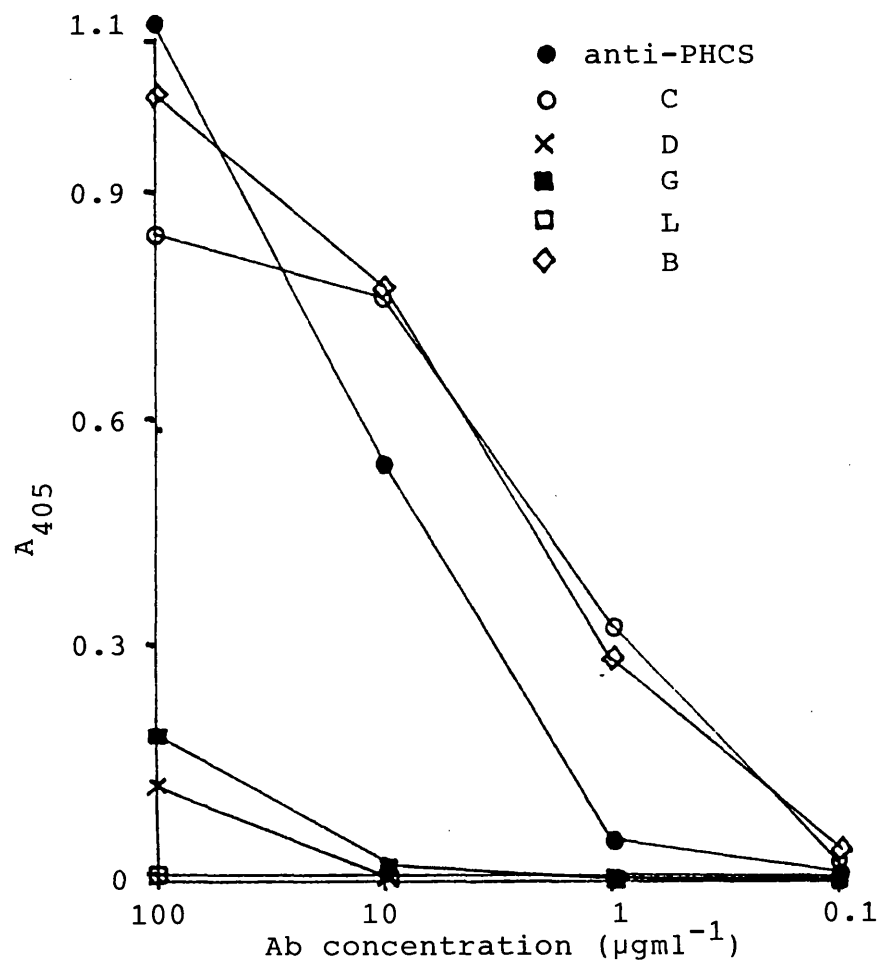


Fig 5.7 Dilution curves for mAbs in ELISA V (poly-L-aspartate pre-coated plate coated with PHCS at pH7.3). Mouse anti-PHCS serum ● was used at dilutions of 1/50, 1/500, 1/5000 and 1/5x10⁴

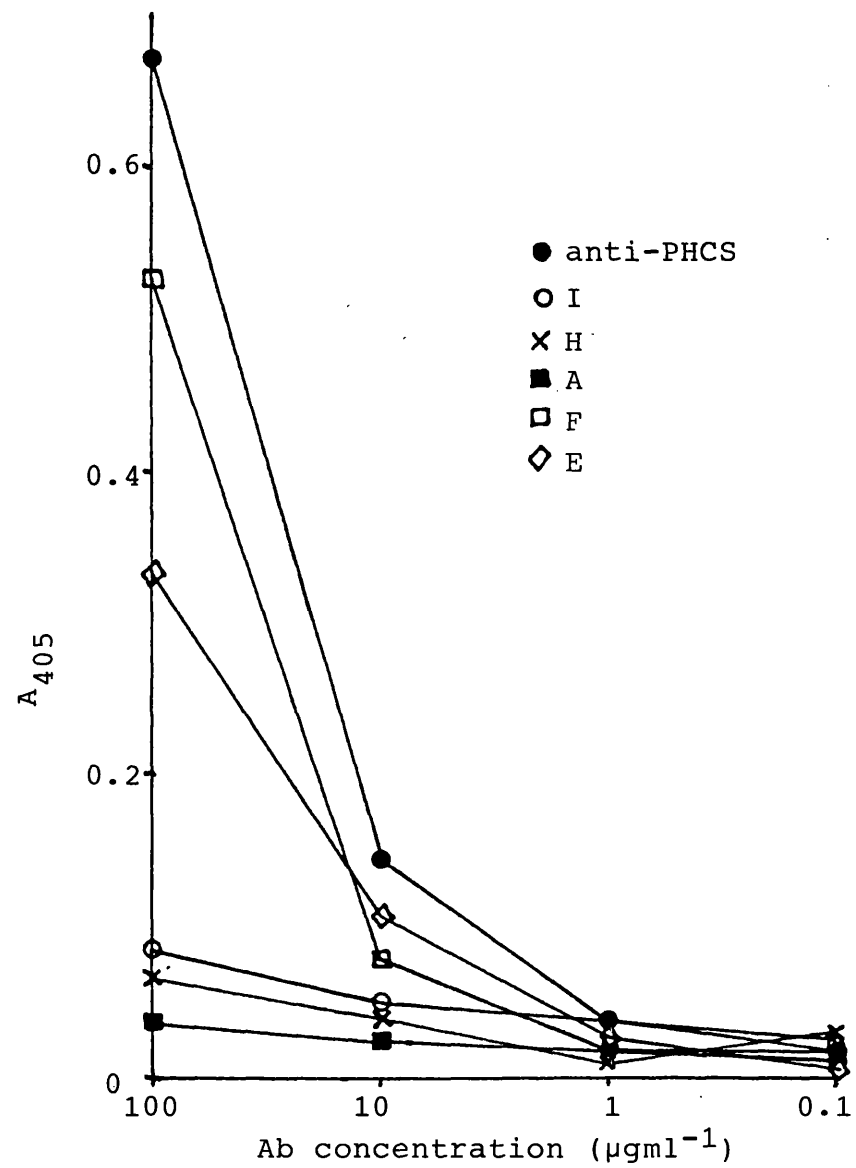
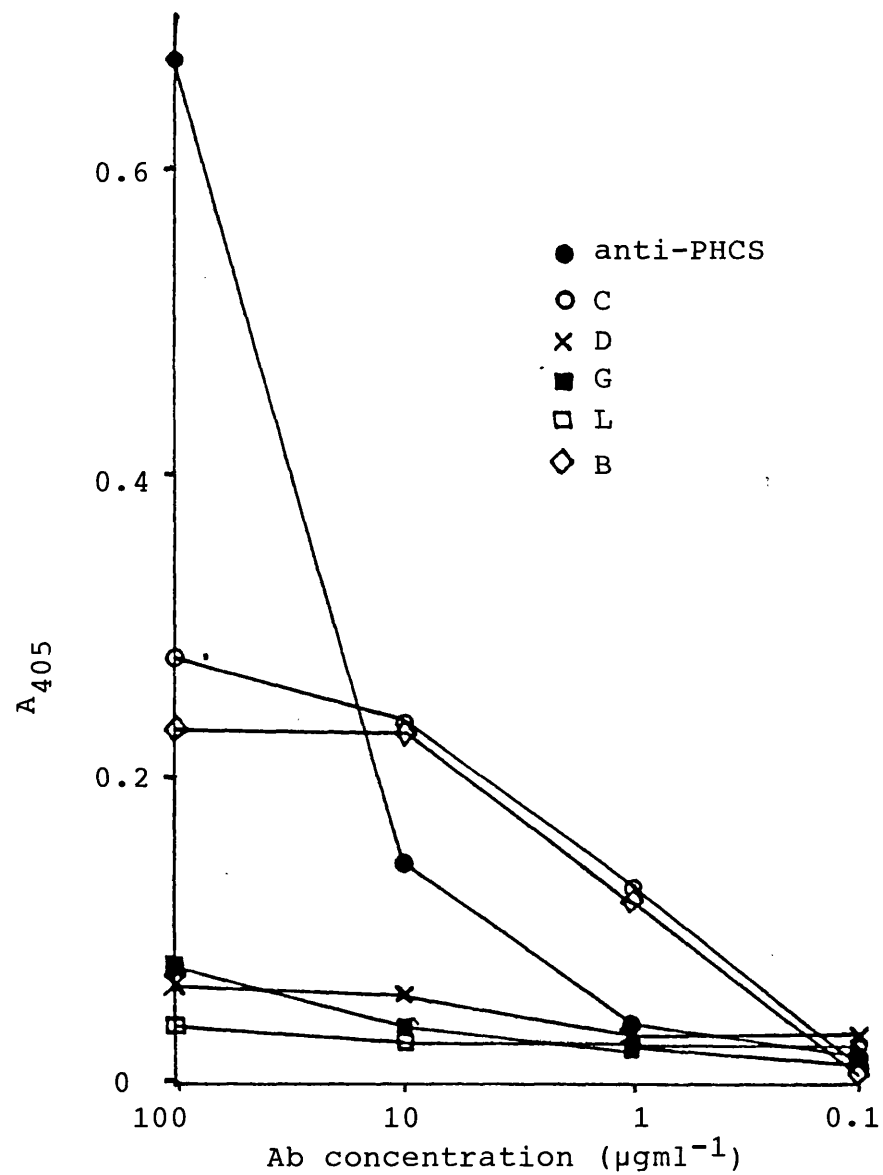


Fig 5.8 Dilution curves for mAbs in ELISA VI (poly-L-lys pre-coated plate coated with PHCS at pH 4.2). Mouse anti-PHCS serum ● was used at dilutions of 1/50, 1/500, 1/5x10³ and 5x10⁴. Substrate incubation was for 1h.

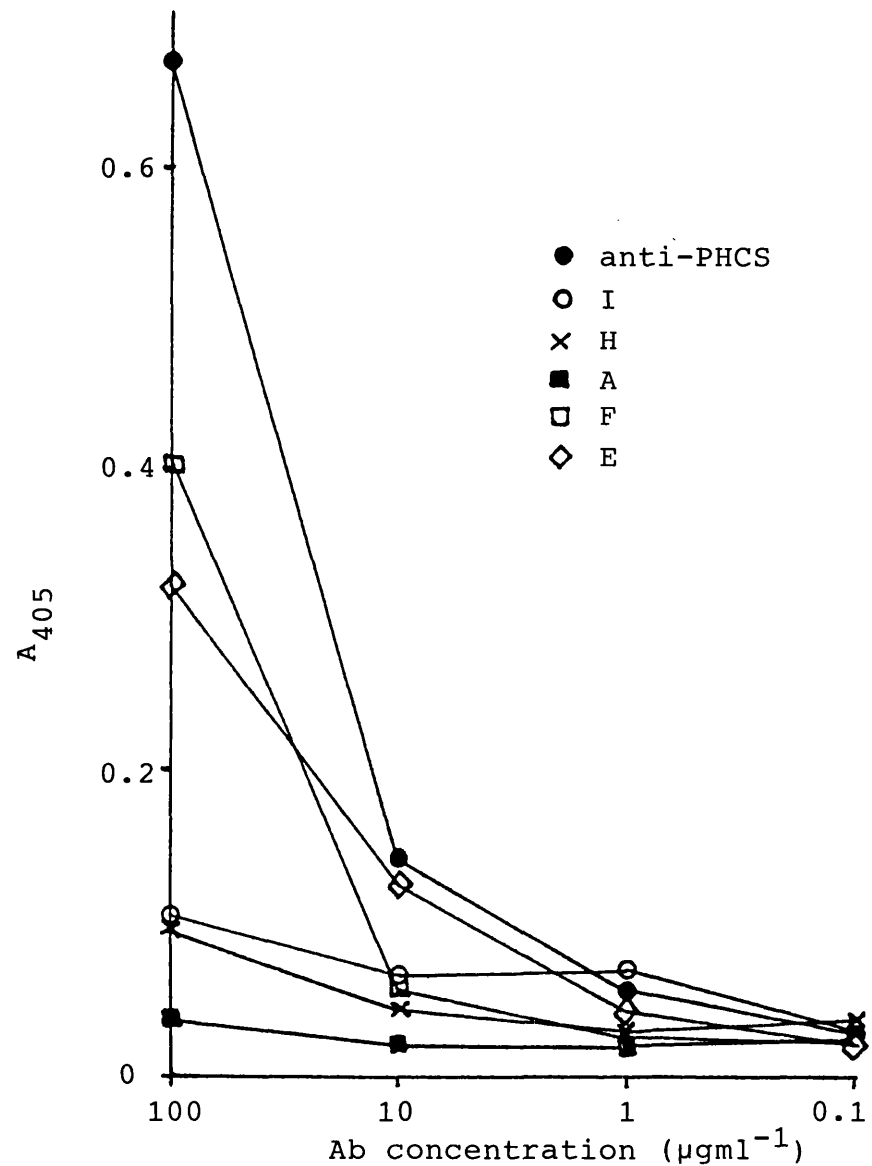
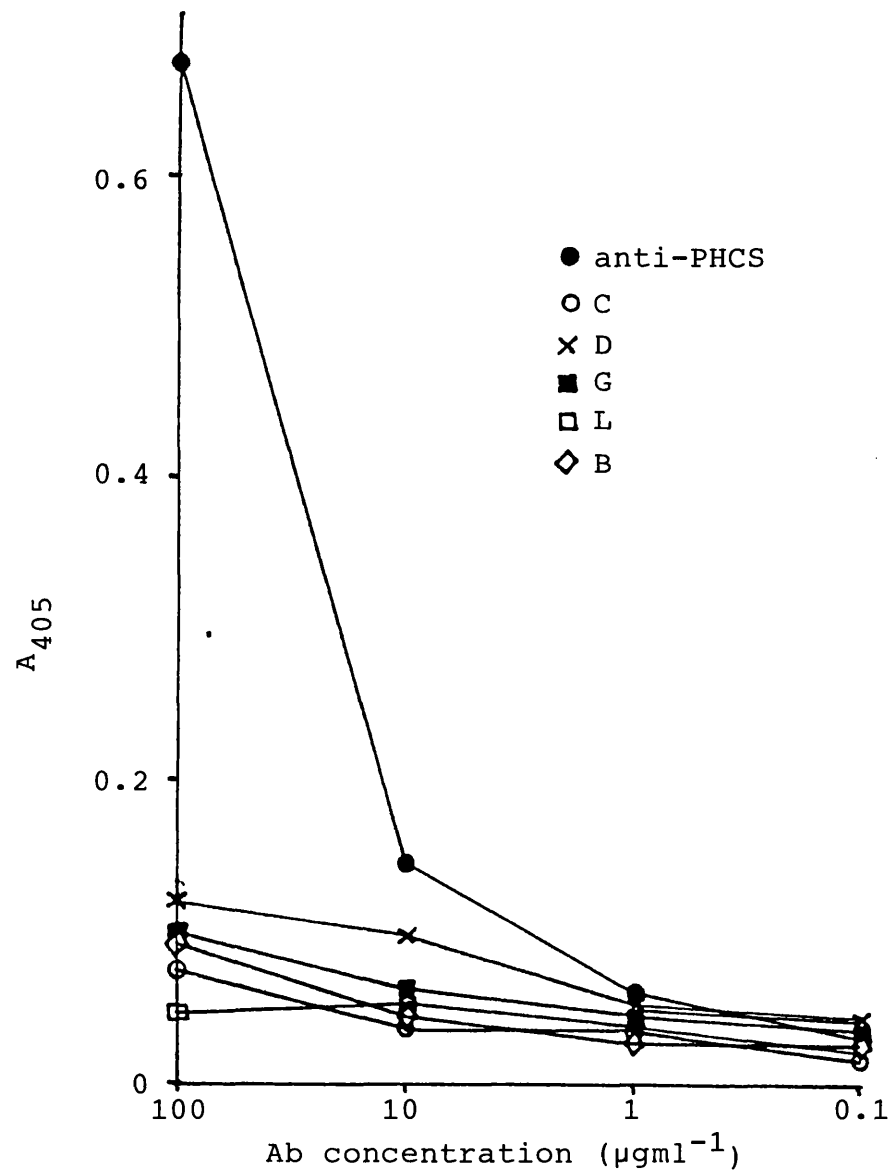


Fig 5.9 Dilution curves for mAbs in ELISA VII (poly-L-lys pre-coated plate coated with PHCS at pH7.3). Mouse anti-PHCS serum ● was used at dilutions of 1/50, 1/500, 1/5000 and 1/5x10⁴) Substrate incubation was for 1h.

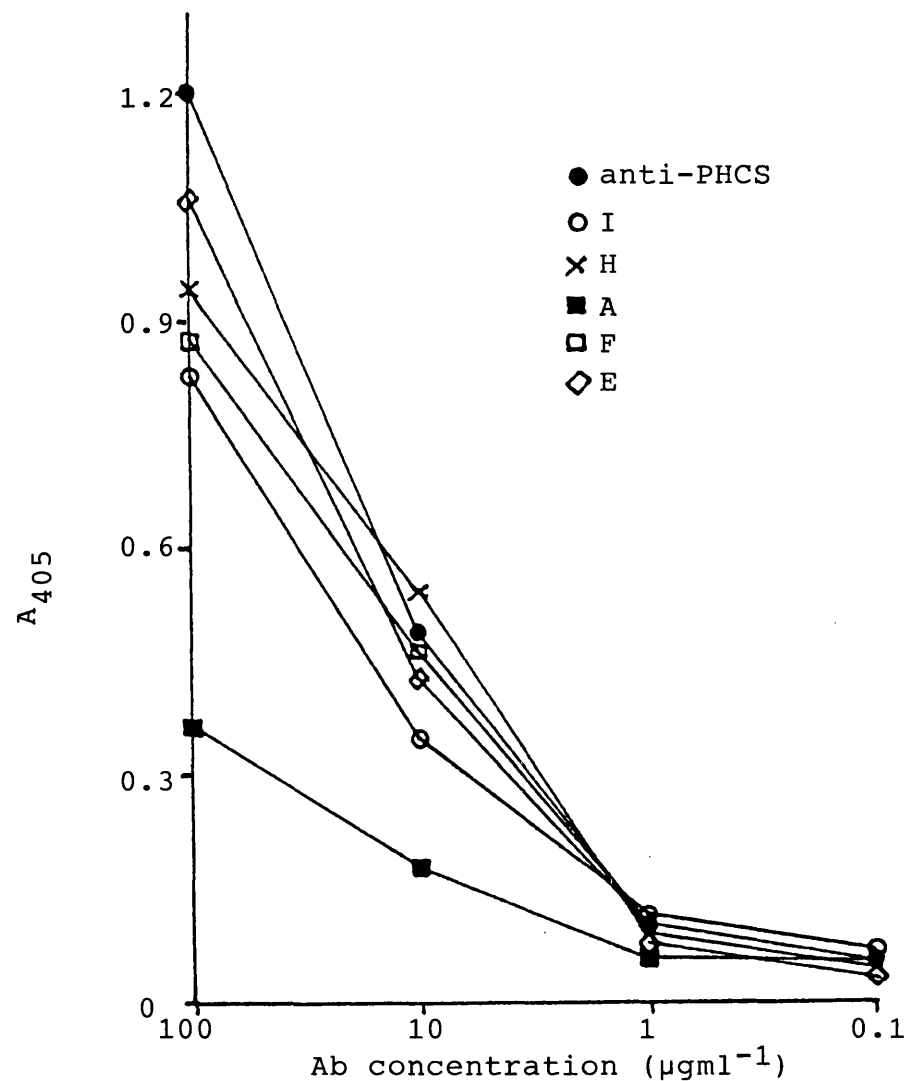
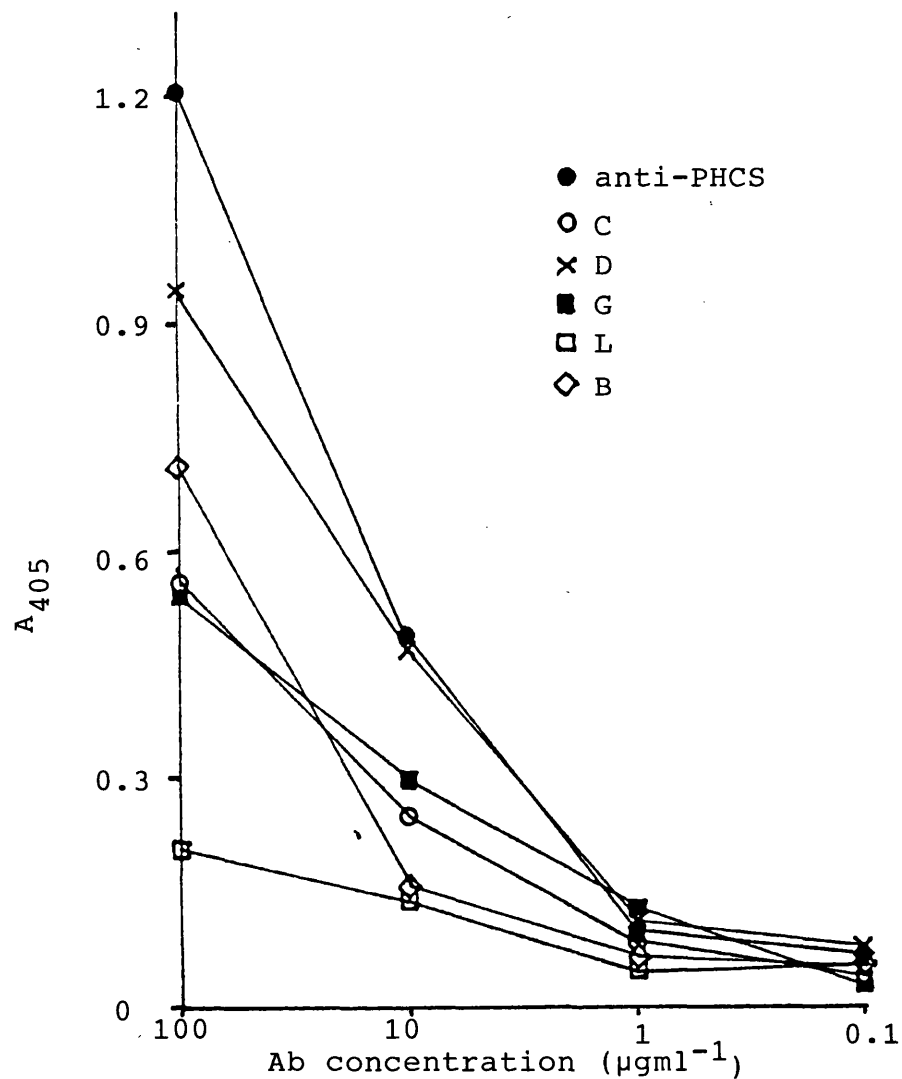


Fig 5.10 Dilution curves for mAbs in ELISA VIII (sandwich assay). Mouse anti-PHCS serum ● was used at dilutions of 1/50, 1/500, 1/5x10³ and 1/5x10⁴.

Substrate incubation was for 30 min.

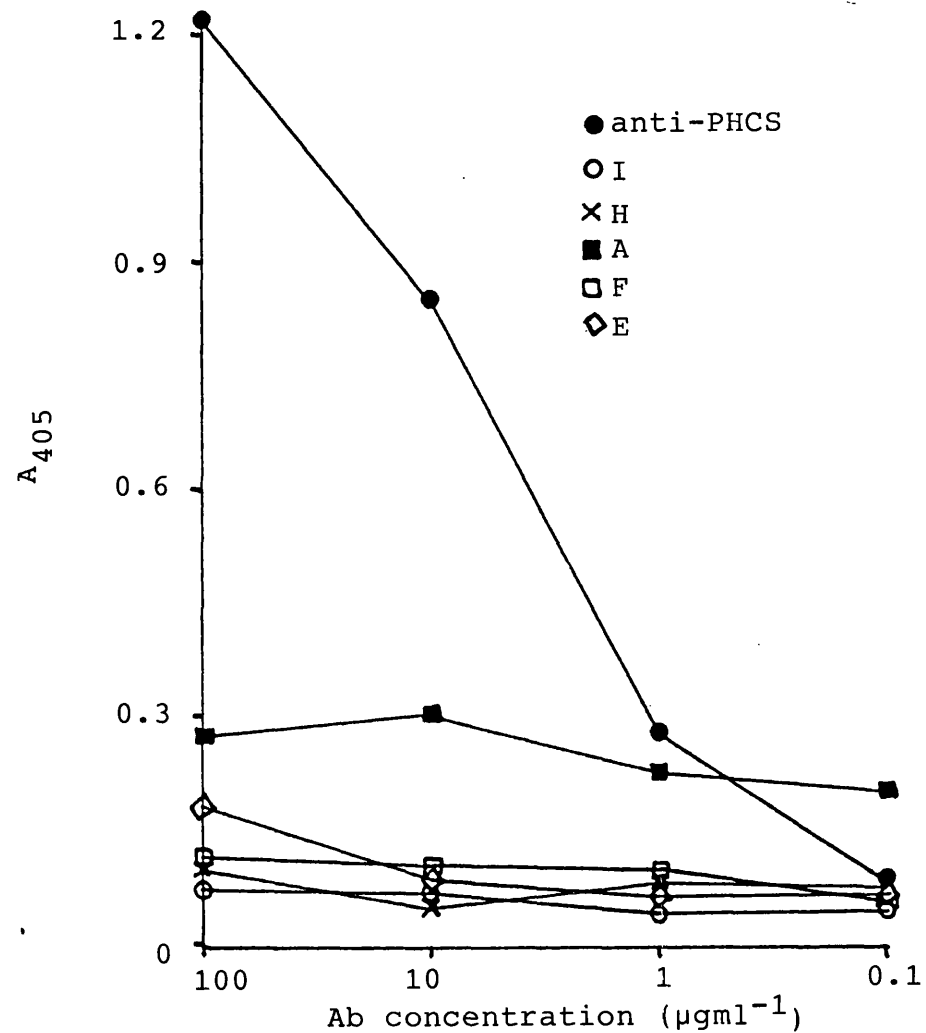
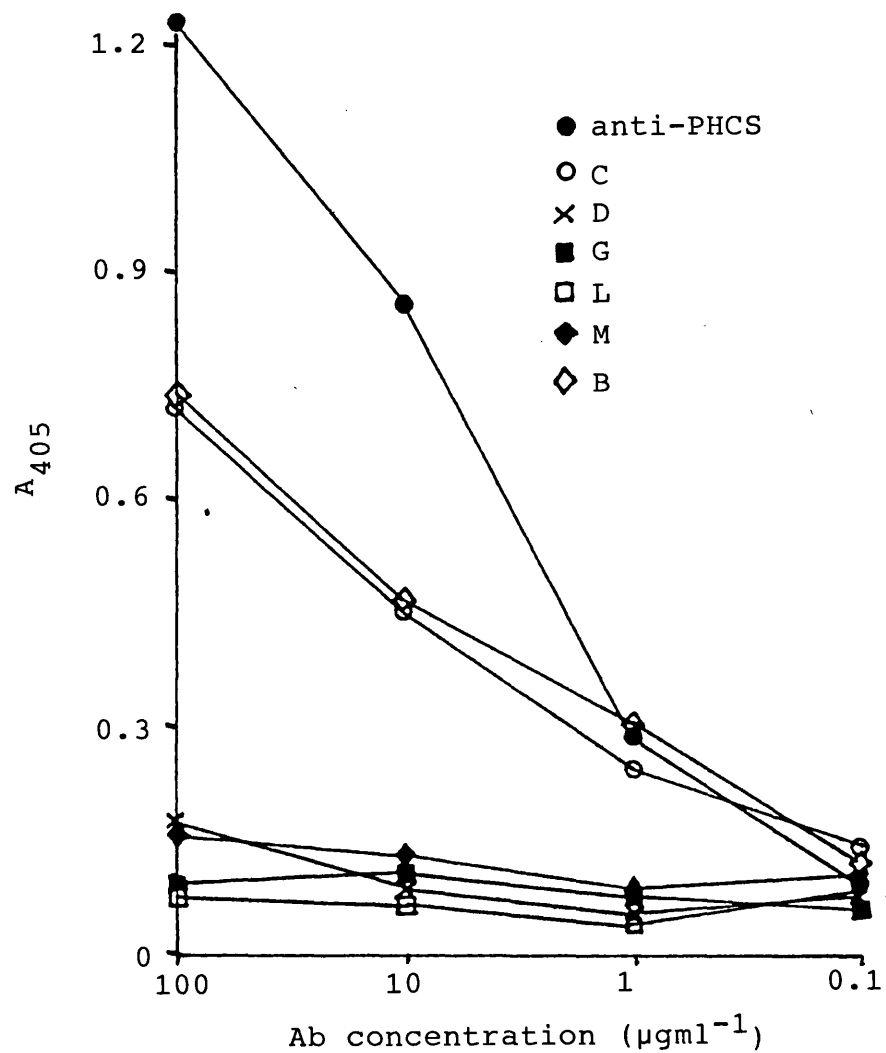


Fig 5.11 Dilution curves for mAbs in immunodot-blot assay IX (PHCS coated directly at pH9.8). Mouse anti-PHCS serum ● was used at dilutions of 1/50, 1/500, 1/5000 and 1/5x10⁴. Substrate incubation was for 35 min

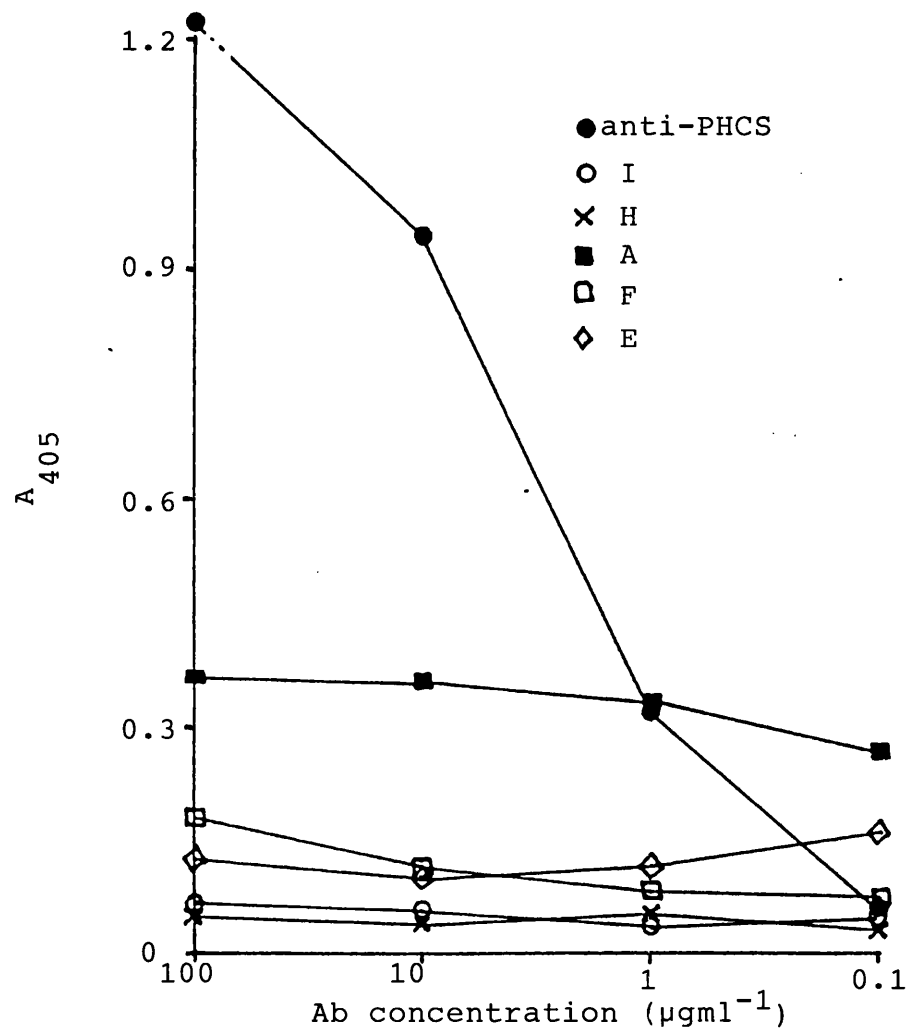
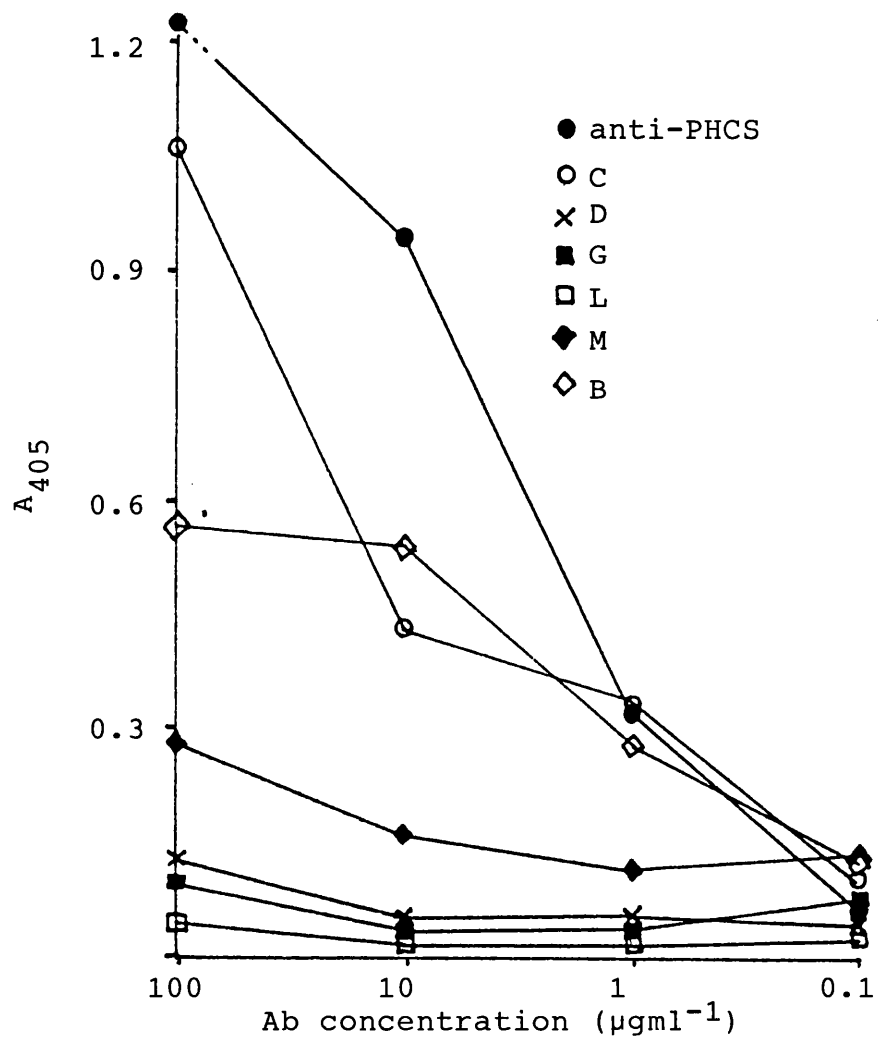


Fig 5.12 Dilution curves for mAbs in immunodot blot assay X (PHCS coated directly at pH7.3). Mouse anti-PHCS serum ● was used at dilutions of 1/50, 1/500, 1/5000 and 1/5x10⁴. Substrate incubation was for 35 min.

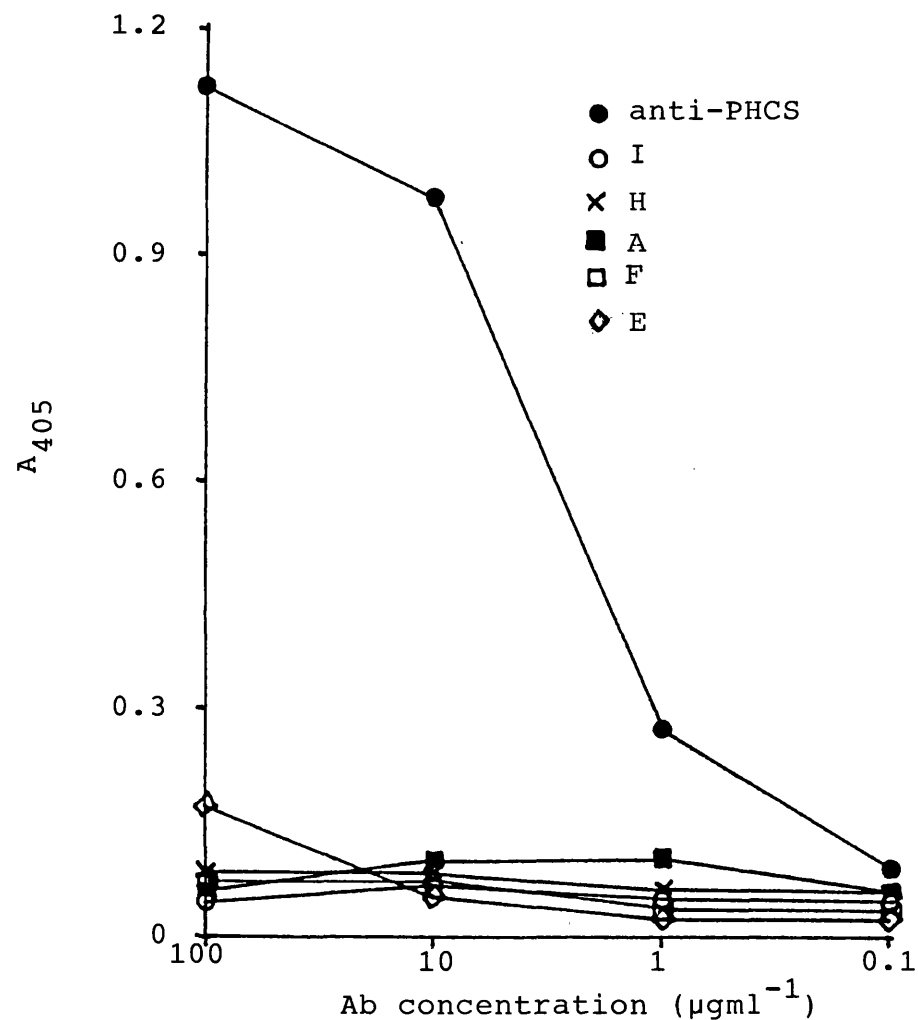
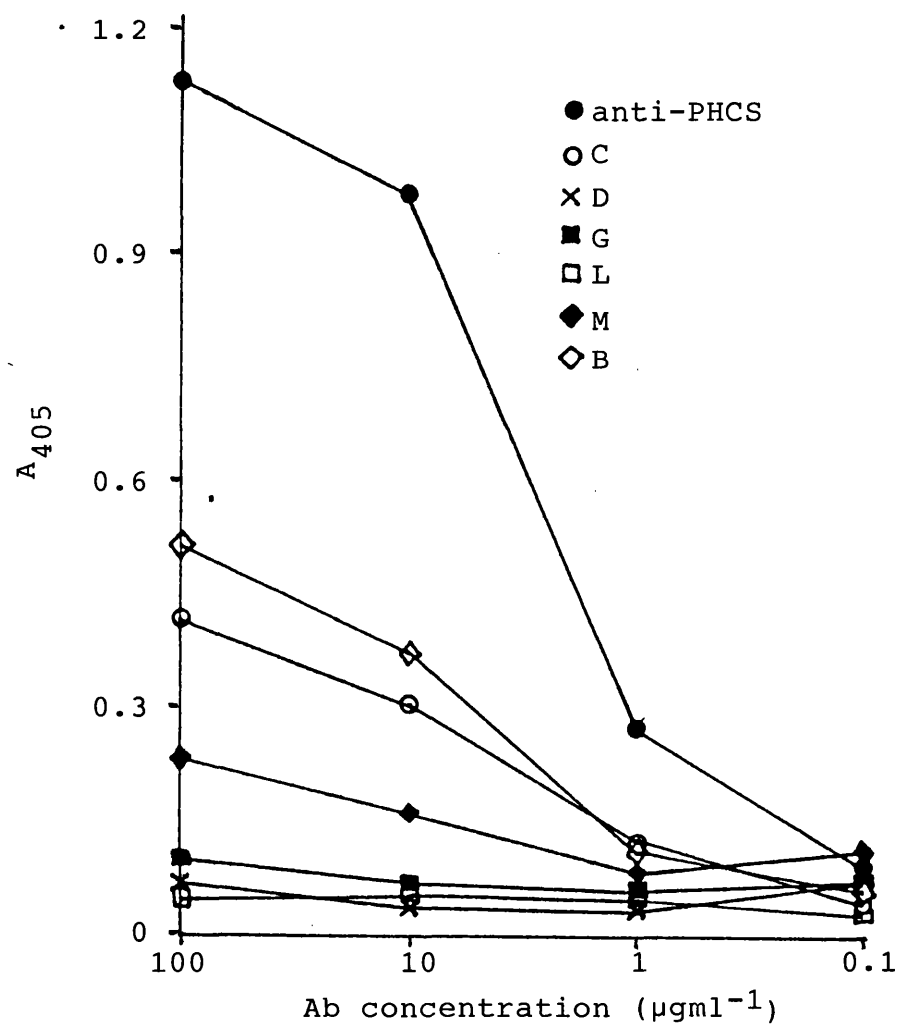


Fig 5.13 Dilution curves for mAbs in immunodot blot assay XI (PHCS coated directly at pH4.2). Mouse anti-PHCS serum ● was used at dilutions of 1/50, 1/500, 1/5000 and 1/5x10⁴. Substrate incubation was for 35 min.

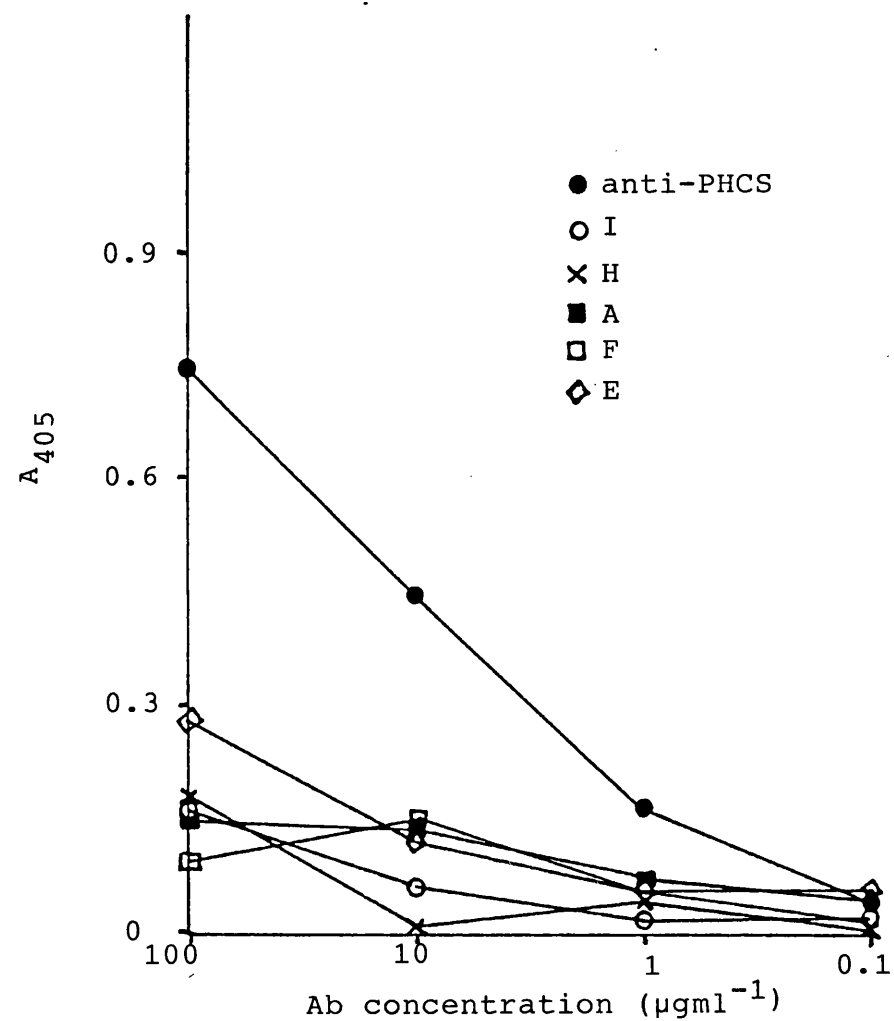
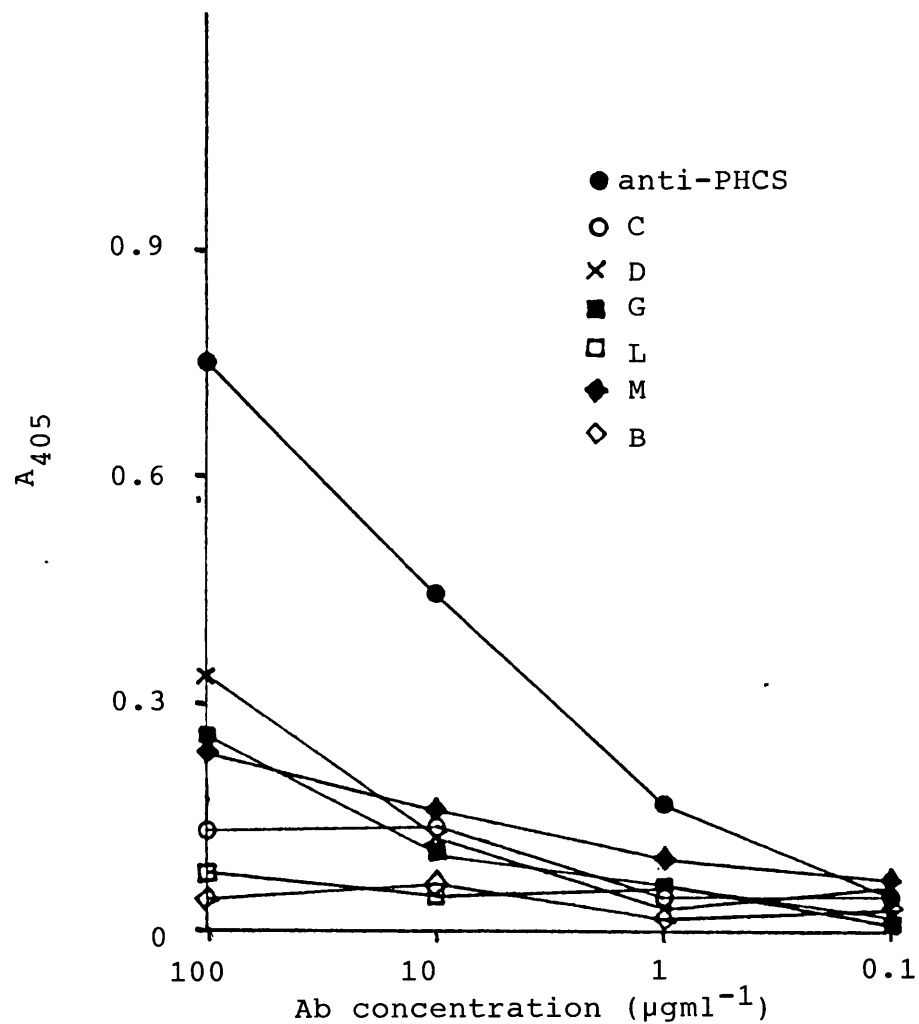
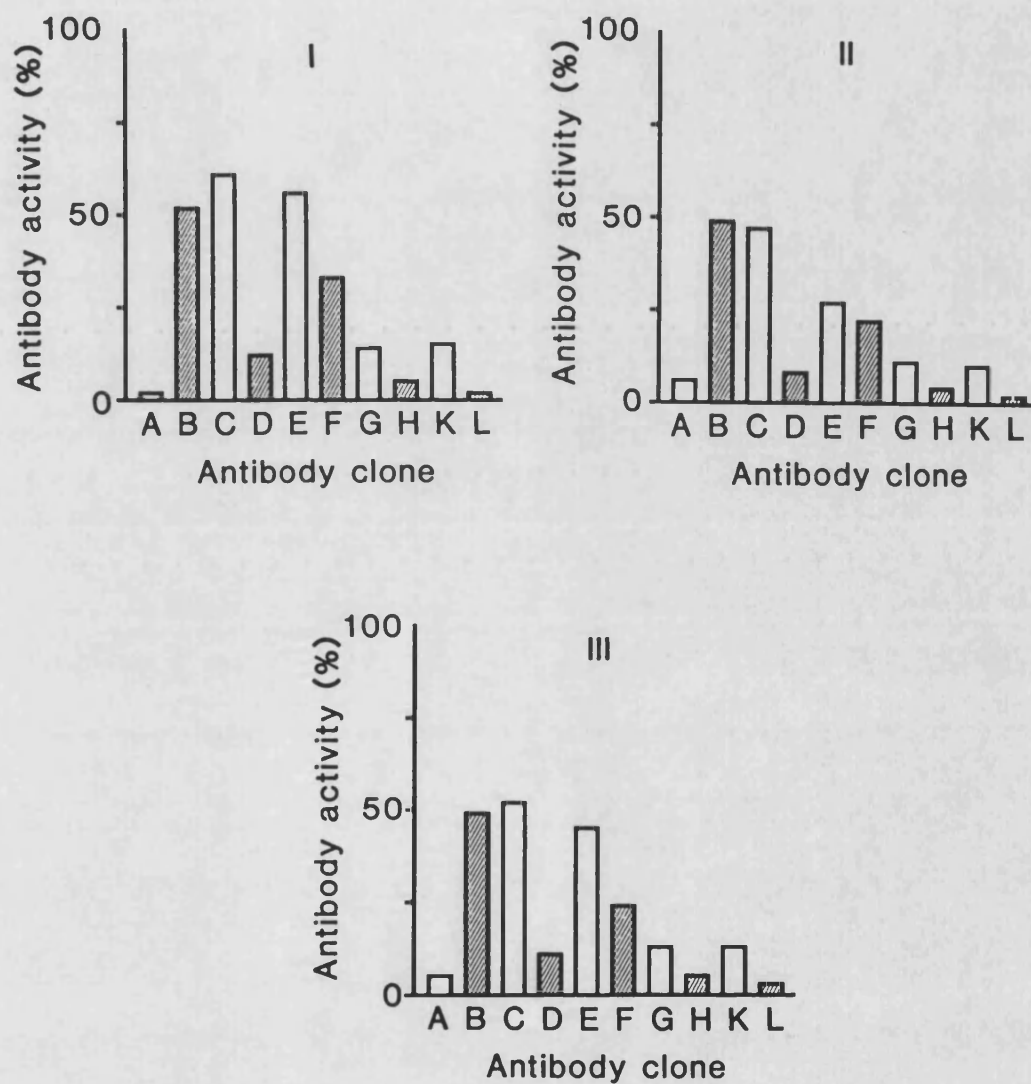


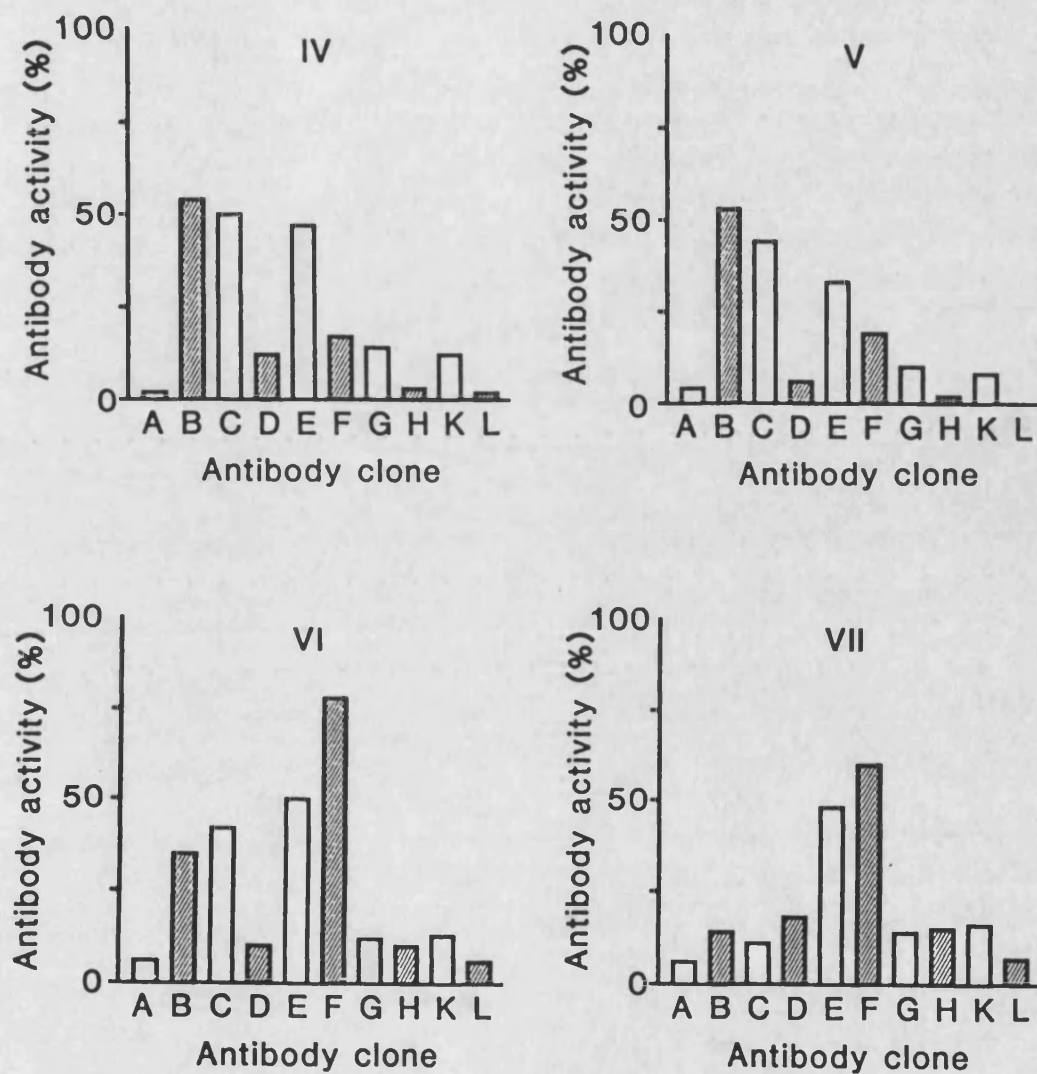
Fig 5.14 Dilution curves for mAbs in immunodot blot assay XII (sandwich assay). Mouse anti-PHCS serum ● was used at dilutions of 1/50, 1/500, 1/5000 and 1/5x10⁴. Substrate incubation was for 30 min.

Fig 5.15 Histograms showing the relative activities of mAbs in assays I, II and III



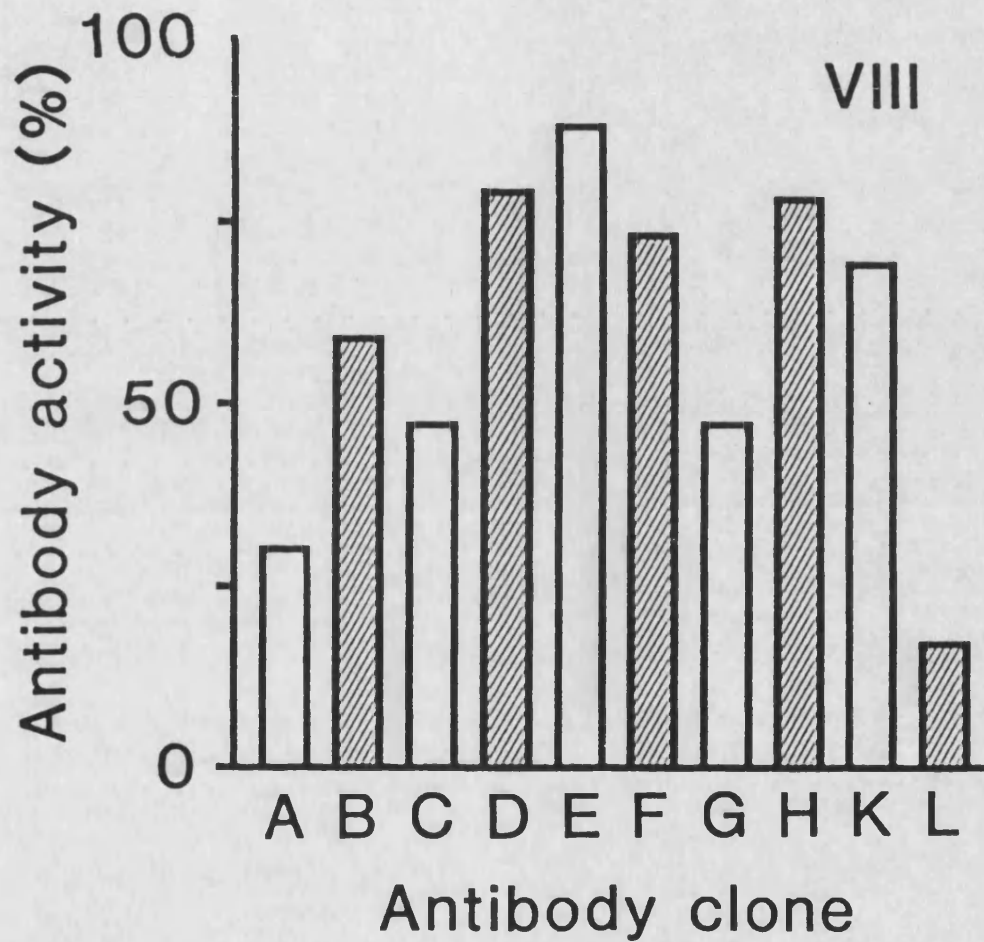
The activity of each mAb (at $100\mu\text{gml}^{-1}$ concentration) is expressed as a percentage of the A_{405} value given by a 1/50 dilution of mouse anti-PHCS serum.

Fig 5.16 Histograms showing the relative activities of mAbs in assays IV - VII.



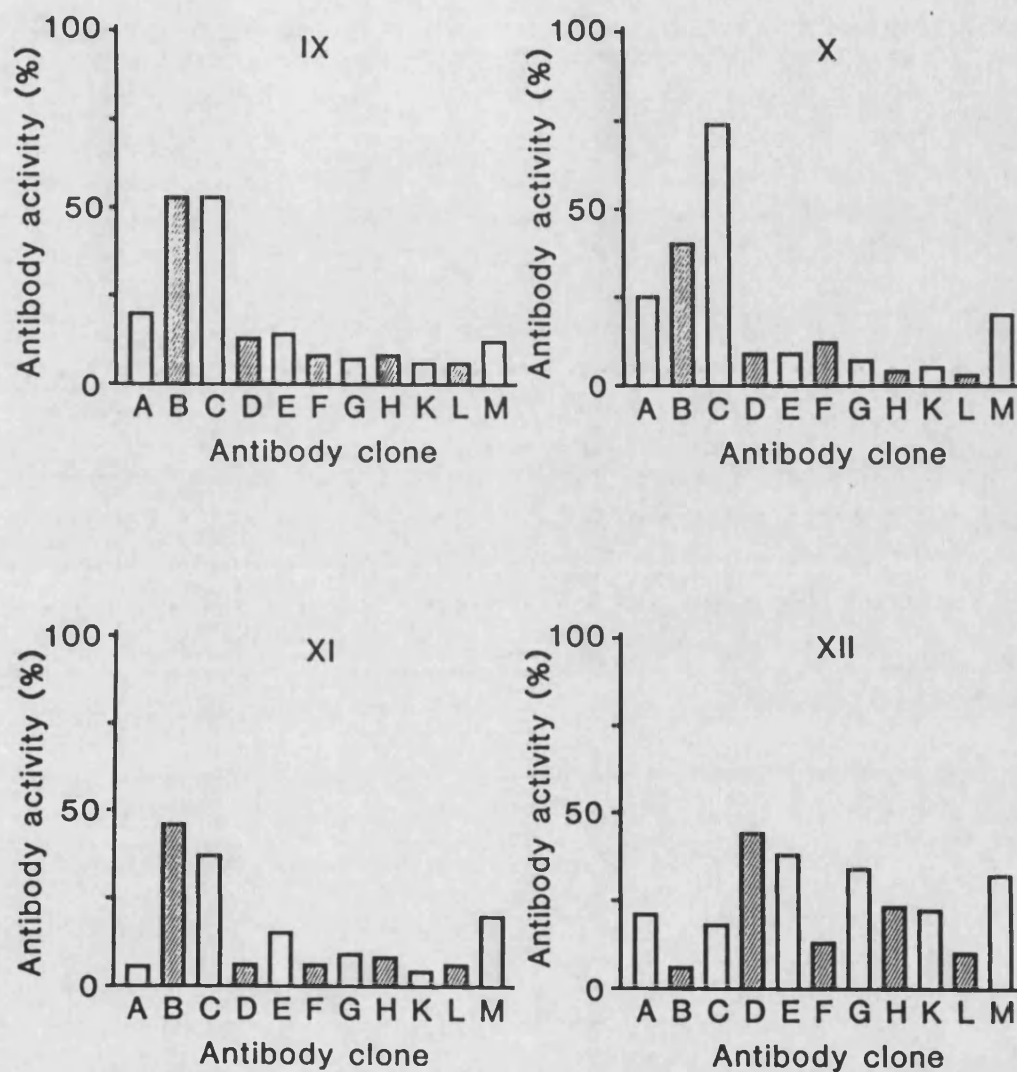
The activity of each mAb (at $100\mu\text{gml}^{-1}$ concentration) is expressed as a percentage of the A_{405} value given by a 1/50 dilution of mouse anti-PHCS serum.

Fig 5.17 Histogram showing the relative activities of mAbs in the sandwich ELISA, assay VIII



The activity of each mAb (at $100\mu\text{gml}^{-1}$ concentration) is expressed as a percentage of the A_{405} value given by a 1/50 dilution of mouse anti-PHCS serum.

Fig 5.18 Histograms showing the relative activities
of mAbs in assays IX - XII



The activity of each mAb (at $100\mu\text{gml}^{-1}$ concentration) is expressed as a percentage of the A_{405} value given by a 1/50 dilution of mouse anti-PHCS serum.

of positive control mouse antiserum and all mAbs gave >17% of the control response.

When the immunodot blot assay (XI) was used, mAbs B, C and M responded strongly (figs 5.13 and 5.18). When the pH of the coating buffer was varied (assays IX and X) these clones remained positive, and in addition clone A was detected (figs 5.11, 5.12 and 5.18). All mAbs were detected using the sandwich immunodot blot (assay XII, figs 5.14 and 5.18), although B and C were less reactive than in the direct coating immunoblots. In general, the immunodot blot assays were much less sensitive than the ELISA methods.

5.4 'Open' and 'Closed' conformations of PHCS used in sandwich ELISAs

Two variations of sandwich ELISAs (assay VIII) were performed. In one, the enzyme was incubated with a rabbit anti-PHCS pre-coated plate as in section 3.18. In the other, the enzyme ($10\mu\text{gml}^{-1}$) was pre-incubated with oxaloacetate (30mM) for 2 min prior to the solution being incubated with a rabbit anti-PHCS pre-coated EIA plate as in section 3.18.

The relative reactivities of the mAbs were compared using the two sandwich ELISAs (figs 5.19 and 5.20). The results are presented in the form of histograms of the relative signals given by mAbs used at a concentration of $100\mu\text{gml}^{-1}$ (fig 5.21).

From these data it can be seen that a substantial drop occurs in the relative reactivities of all the mAbs, except mAb E, when comparing a

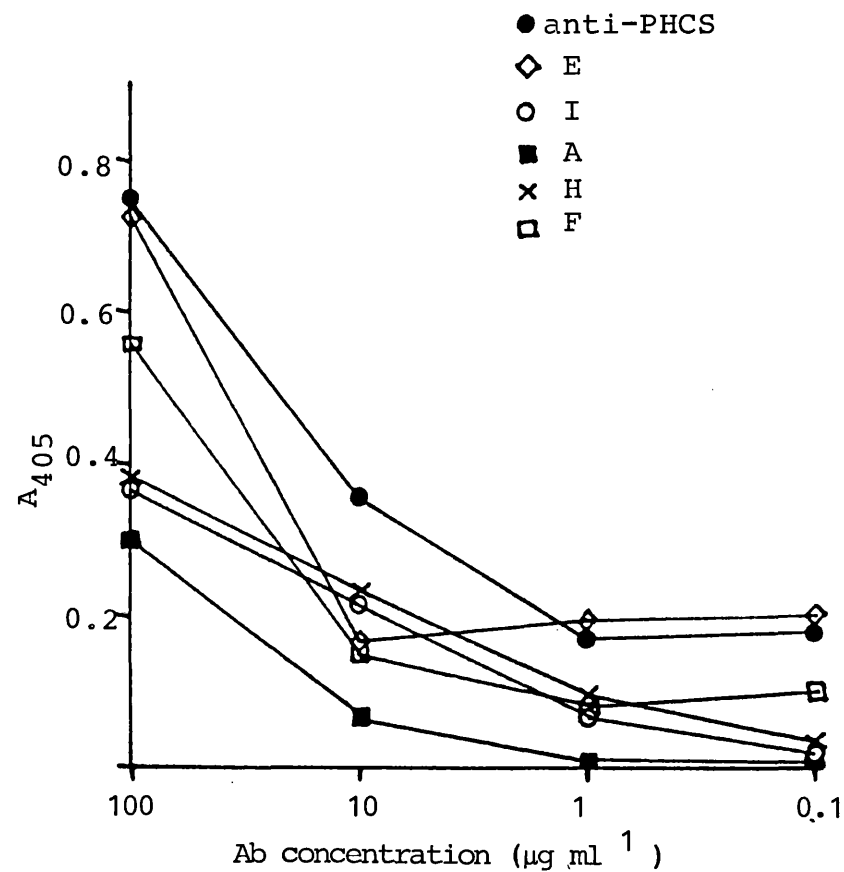
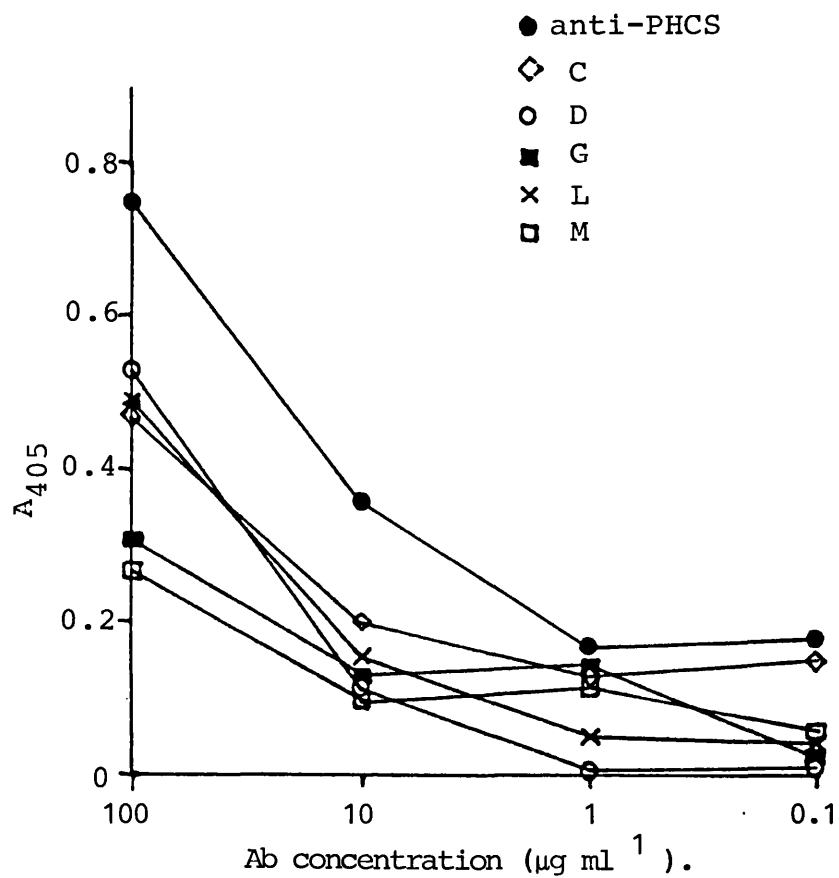


Fig 5.19 Dilution curves of mAbs in a sandwich ELISA where PHCS was not pre-treated.

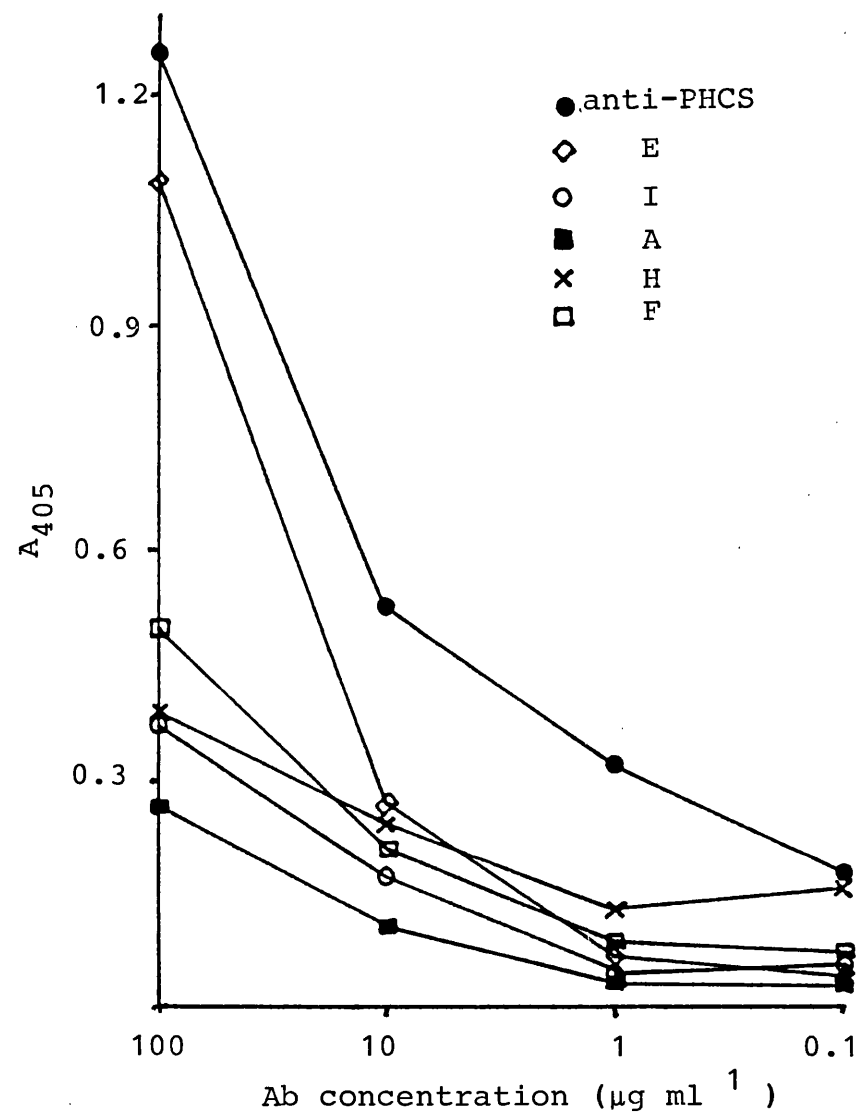
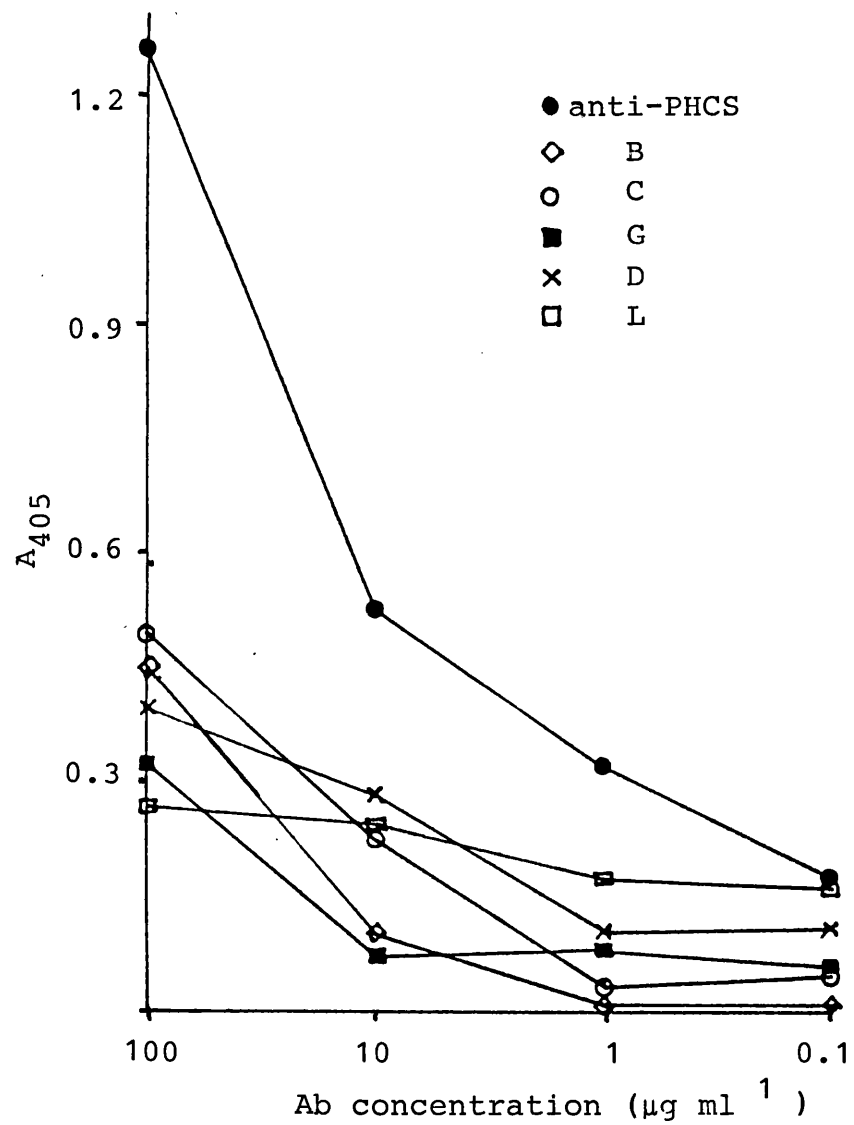
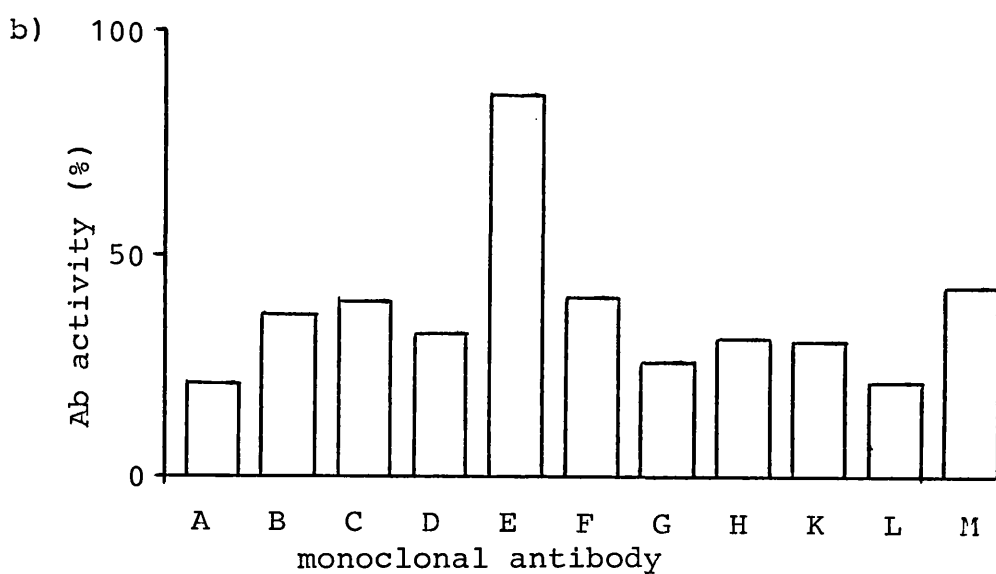
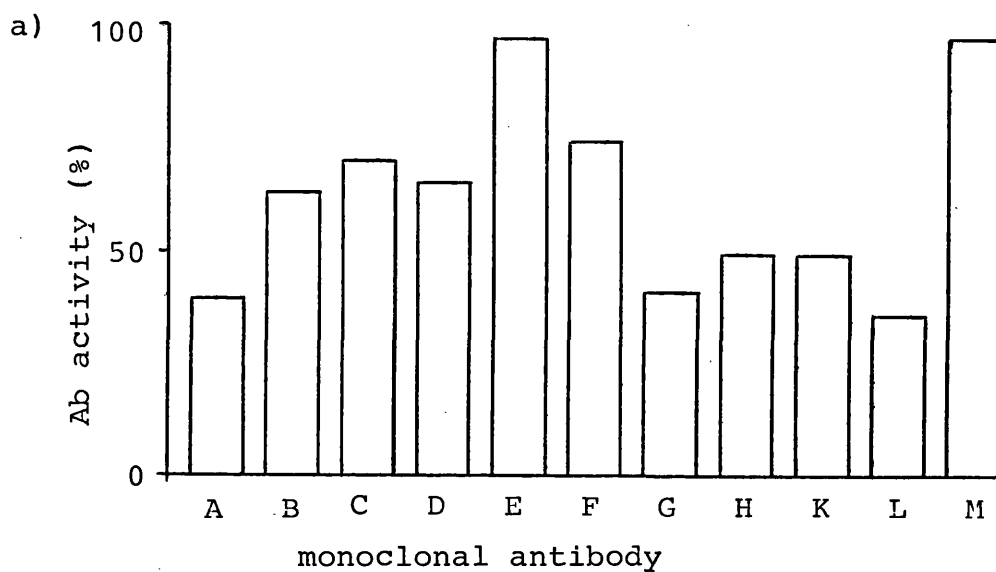


Fig.5.20 Dilution curves of mAbs in a sandwich ELISA where PHCS was pretreated with oxaloacetate.

Fig.5.21 Histograms showing the relative activities of mAbs in sandwich ELISAs in which PHCS is presented in the 'open' and 'closed' forms



The activity of each mAb (at a concentration of $100 \mu\text{gml}^{-1}$) is expressed as a percentage of the A_{405} value given by a $\frac{1}{50}$ dilution of mouse anti-PHCS serum.

a) PHCS untreated prior to coating.

b) PHCS pre-incubated with oxaloacetate prior to coating.

sandwich ELISA which includes oxaloacetate, with one that does not. The relative signal given by mAb E in the ELISA with oxaloacetate present is 89% of that seen in the ELISA without oxaloacetate. This is only a small drop. The relative signals given by all the other mAbs in the ELISA with oxaloacetate present are only 43-63% of their respective signals in the ELISA without oxaloacetate.

5.5 The effect of SDS and 2-mercaptoethanol on the presentation of PHCS in sandwich ELISAs

Four sandwich ELISAs were performed essentially as described in section 3.18, differing only in the ways in which the PHCS was treated prior to incubation with a rabbit anti-PHCS pre-coated EIA plate. The different treatments are listed below.

- 1) PHCS incubated for 5 min in PBS
- 2) PHCS boiled for 2 min in 4% (w/v) SDS/PBS, and subsequently cooled.
- 3) PHCS boiled for 2 min in 10% (w/v) 2-mercaptoethanol/PBS, and subsequently cooled.
- 4) PHCS boiled for 2 min in SDS/PAGE sample buffer (section 3.24) and subsequently cooled.

The relative reactivities of mAbs were compared at a concentration of 100µg/ml. A control normal mouse serum was used at a dilution of 1/100. For convenience the enzyme-conjugate used in these ELISAs was horse radish peroxidase-conjugated goat anti-mouse IgG

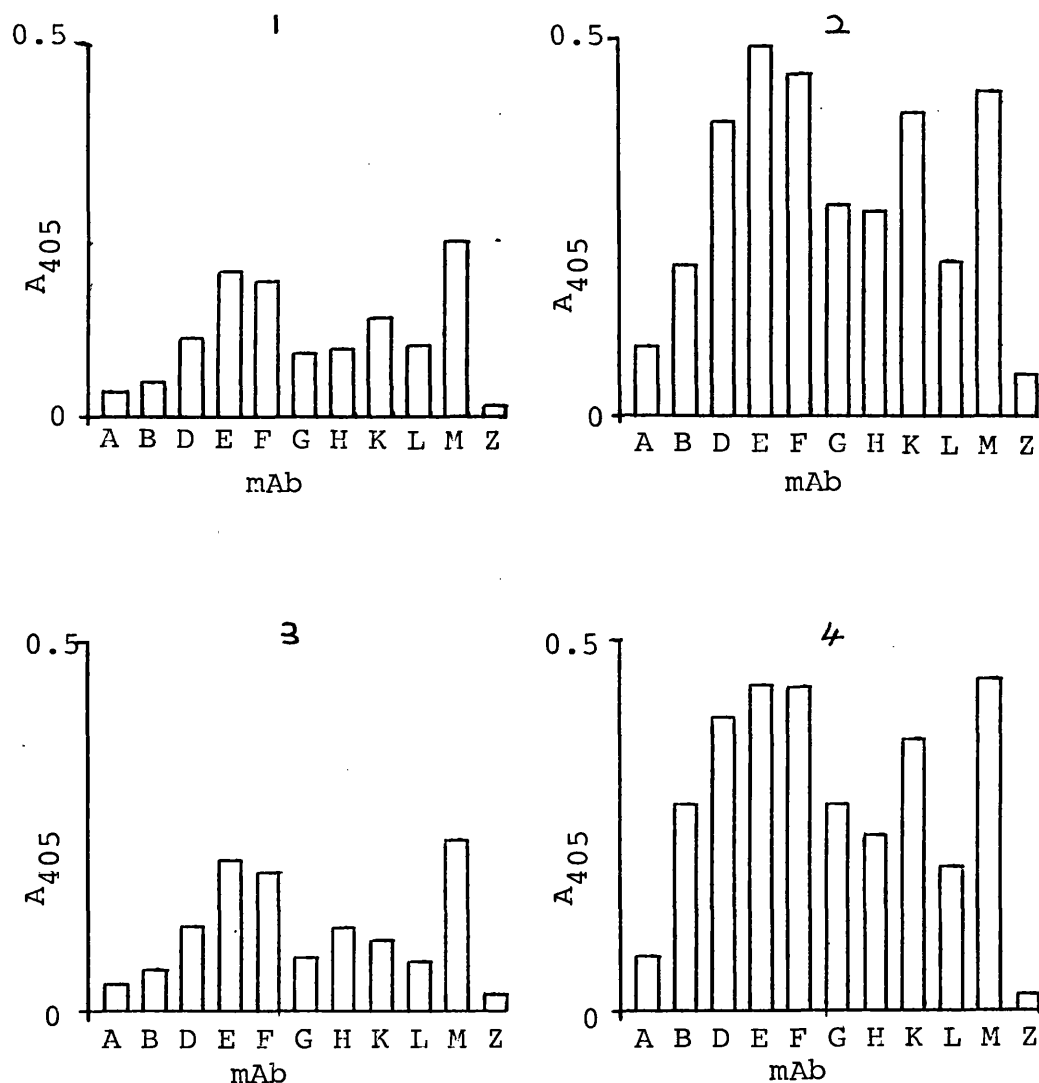
(diluted 1/1000). Incubation with substrate (0.01% 3,3'-5,5'-tetramethylbenzidine, 1% dimethylsulphoxide in 0.1M sodium acetate/citric acid buffer pH6.0) was continued for 30 min, after which the reaction was stopped by addition of 50 μ l of 2M sulphuric acid.

The results are shown in figs 5.22 as histograms representing the final absorbance for each mAB.

From the data it can be seen that boiling PHCS in 2-mercaptoethanol has no effect on the reactivity of mAbs, when compared to an unaltered sandwich ELISA (fig 5.22). However, when the enzyme is boiled in SDS, or SDS PAGE sample buffer, the eventual reactivities of all mAbs are seen to increase substantially when compared to the unaltered sandwich ELISA.

Further consideration of these results suggested the need for a series of controls in which antibody-coated EIA plates were treated with SDS or SDS + 2-mercaptoethanol in the absence of PHCS. However, these experiments could not be completed since the MAb reagents were no longer available. It is unlikely that the differences in reactivity shown in Fig 5.22 are due to increased nonspecific binding of MAb to rabbit anti-PHCS; altered antigenicity of PHCS following exposure to SDS remains the most likely explanation of these results (see p 132).

Fig.5.22 Histograms showing the relative activities of mAbs in sandwich ELISAs in which the PHCS has undergone various pre-treatments



The activity of each mAb (at a concentration of $100\mu\text{gml}^{-1}$) is expressed as the A_{405} value reached following a 30min incubation with substrate. A control normal mouse serum (Z) was used at a dilution of $\frac{1}{100}$. The pre-treatments of PHCS, 1-4 are as described in the text (section 5.5).

5.6 Discussion

In solid-phase immunoassays, antigen presentation plays an important role in the reactivity of mAbs. Solid-phase systems such as ELISAs and immunodot-blot assays are widely used in the screening of mAbs (Engvall, 1980; Hawkes et al, 1982; DeBlas and Cherwinski, 1983). However, variation in the reactivity of mAbs has been noted between different ELISAs and liquid-phase assay systems (Al Moudallal et al, 1984; McCullough et al, 1985). It has been suggested that some antibodies selected by direct ELISA are specific for antigenic sites which are hidden in the native protein, but become exposed once the protein is coated onto a solid support (Friguet et al, 1984).

I have attempted to choose a method which ensures that mAbs are not lost due to failure to detect certain specificities in the initial screen. For this reason, I have compared 12 assay systems for their ability to detect the members of a library of mAbs prepared against PHCS.

In ELISA systems using a poly-L-aspartate spacer (assays IV and V), the antigen is probably immobilized via free carboxylic acid groups on the spacer. When the anti-PHCS mAbs were compared, the results obtained in these assays were similar to those from assays where no spacer was used (assays I, II and III). This suggests that the interactions responsible for antigen immobilization are similar in these assays. However, when the mAbs were compared using ELISA

systems with poly-L-lysine as spacer (assays VI and VII) the results differed from those of assays I-V, thus indicating that the antigen is bound in a different orientation, or with an altered conformation, when using spacers carrying positive or negative charges (poly-L-lysine or poly-L-aspartate). This observation was confirmed when assays V and VII were used in the initial screening of colonies from a new fusion (table 5.1). The use of the alternative spacers resulted in the detection of different sets of antibody-producing cultures.

In the sandwich ELISA (assay VIII), antigen is immobilized to a polyclonal antibody-coated solid phase. The results from this assay suggest that antigen presentation is quite different to that obtained in direct ELISAs. The fact that only in assay VIII were all the mAbs detected strongly indicates that in this system antigen is presented in a variety of orientations or conformations, as might be expected from the variety of specificities of anti-PHCS present in a polyclonal antibody preparation.

Comparison of mAbs using immunodot-blot assays (IX, X and XI) showed some differences from the ELISA results. These can be attributed to differences in the mechanism of antigen immobilization between the two systems. Protein binding to nitrocellulose is thought to involve hydrophobic interactions predominantly, while binding to polystyrene is thought to involve ionic interactions. The sandwich dot assay (XII) gave positive results with all mAbs.

This finding is similar to the sandwich ELISA result and presumably reflects similarities in the mechanism of antigen immobilization in these two systems, although the sandwich ELISA is more sensitive.

PHCS with bound oxaloacetate has been shown to exist in a 'closed' form, whereas the free enzyme exists in an 'open' conformation (Wiegand et al, 1984; Chothia and Lesk, 1985). The two sandwich ELISAs used in section 5.4 were designed to capture and present PHCS in its 'open' and 'closed' forms respectively. MAb E was able to react well in both ELISA systems. This may indicate that its binding site on the enzyme is altered very little between the 'open' and 'closed' forms of the enzyme. Conversely, the binding of the remaining mAbs was greatly reduced when the enzyme was presented in the 'closed' form, that is, with oxaloacetate bound. This suggests that the conformations of the binding sites of these mAbs may be substantially altered as the enzyme changes from an 'open' to a 'closed' form, or that oxaloacetate blocks the recognition of the Abs for the epitope.

SDS is a detergent and protein denaturing agent. In the sandwich ELISAs in which the PHCS was boiled in SDS prior to its immobilization to a polyclonal antibody-coated solid phase, the reactivity of all the mAbs tested was increased. This may be due to the SDS denaturing the PHCS and freeing epitopes for second antibody binding, or by its increasing the ability of PHCS to bind polyclonal serum coated

solid supports, thus increasing the epitope density presented.

This work emphasizes the importance of antigen presentation effects in solid phase ELISA, and immunodot-blot assays. The results show that sandwich assays, in which the antigen is immobilized in a variety of orientations using polyclonal antibodies, are capable of detecting mAbs with a wide range of specificities. This is in contrast to direct systems, whether a spacer is used or not, each of which detect a set of mAbs of more restricted specificity. This work suggests that sandwich assays should be used in preference to direct assays for initial screening to produce a library of mAbs with a wide range of possible specificities. Further, the work of using SDS, and oxaloacetate, to alter the form of the antigen presented by sandwich ELISAs emphasizes the importance of antigen presentation.

CHAPTER SIX Peptide mapping of mAbs

	Page
6.1 Limited proteolysis of PHCS using chymotrypsin, trypsin and subtilisin.	135
6.2 Peptide mapping of mAbs to proteolytic fragments of PHCS.	137
6.3 Further peptide mapping of mAbs to a tryptic digest of PHCS.	142
6.4 Cleavage of asp-pro bonds by dilute acid.	147
6.5 Peptide mapping of mAbs to acid cleaved fragments of PHCS.	147
6.6 MAb immunoabsorbent chromatography performed on PHCS fragmented at asp-pro bonds.	154
6.7 Prediction of the products formed by cleavage of PHCS at asp-pro bonds.	158
6.8 Peptide mapping of mAbs to PHCS fragmented at asn-gly bonds.	160
6.9 Discussion.	162

6.1 Limited proteolysis of PHCS using chymotrypsin trypsin and subtilisin

Limited proteolysis of PHCS was performed as described in section 3.30. For each of the three proteolytic enzymes used, an initial time course digest was performed. Proteolysis was performed for 10s, 2 min, 5 min, 10 min, 30 min and 2h, after which the reactions were stopped and the digests were run on 8-12% gradient SDS PAGE using the Laemmli method (section 3.23i).

a) Chymotrypsin: The results of a time course digest using a PHCS (subunit): chymotrypsin ratio of 500:1 are shown in fig 6.1. The molecular weights of the fragments were read from a graph of $\log_{10} \%T$ versus $\log_{10} M_{wt}$ plotted for the standards used in track 7. $\log_{10} \%T$ was calculated from the formula:

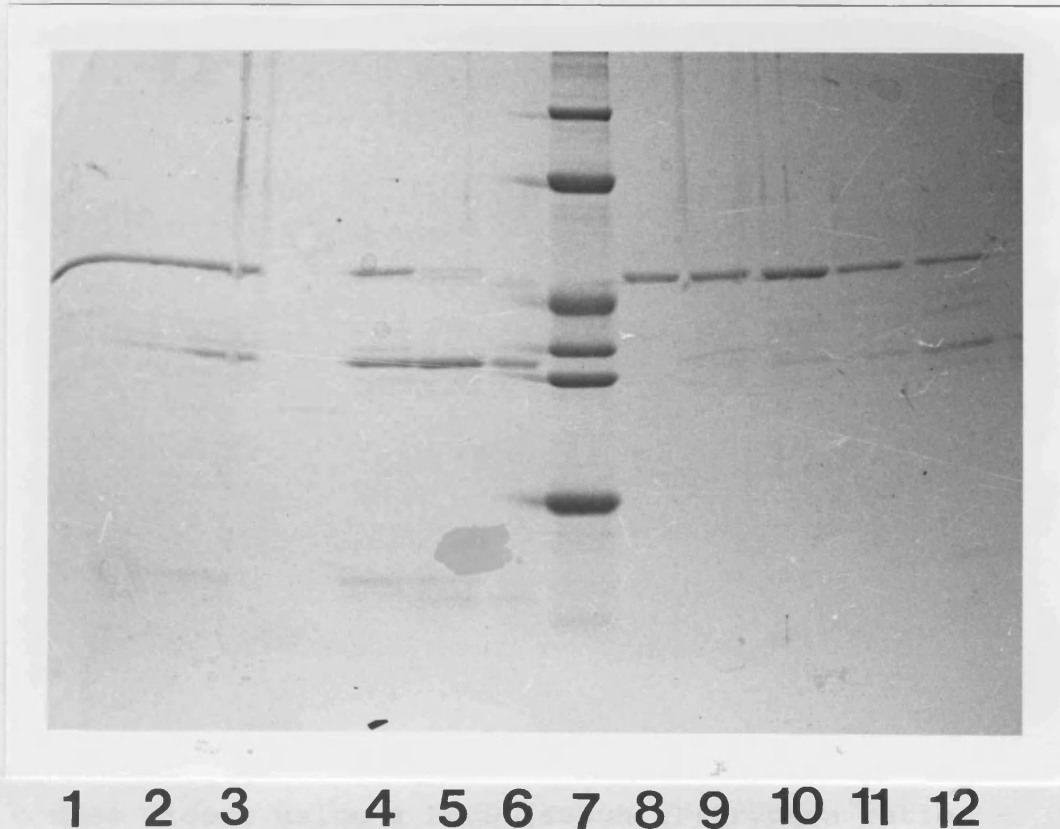
$$\log_{10} \%T = \log_{10} ((D \times \Delta 1\%) + \text{initial gel conc.})$$

where D is the distance travelled through the gel

and $\Delta 1\% = \frac{\text{change in gel concentration}}{\text{gel length}}$

From fig 6.1 it can be seen that following a 10s proteolysis the PHCS subunit is the major band (49K), but faint bands are also present corresponding to molecular weights of 38.5K, 36K and 34K. At 5 min the 34K band has intensified greatly and new bands are visible at 32K, 30K and two bands (<20K). By 30 min the 34K band is the major product, and the native

Fig 6.1 Time dependent proteolysis of PHCS by
chymotrypsin and subtilisin, shown on an
8-12% gradient SDS PAGE gel



Tracks 1-6, PHCS fragments produced by limited proteolysis using chymotrypsin.

Track 7 Molecular weight markers: B-galactosidase (116K), BSA (66K), ovalbumin (45K), glyceraldehyde-3-phosphate dehydrogenase (36K), carbonic anhydrase (29K) and trypsin inhibitor (20K).

Tracks 8-13 PHCS fragments produced by limited proteolysis using subtilisin.

Time course. 10s (tracks 1 and 8), 2 min (tracks 2 and 9), 5 min (tracks 3 and 10), 10 min (tracks 4 and 11), 30 min (tracks 5 and 12), 2h (track 6).

Proteolysis of PHCS was repeated as in

subunit (49K) has nearly all been degraded. Following 2h PHCS is degraded to smaller molecular weight fragments.

b) Subtilisin: The results of a limited time course digest using a PHCS (subunit):subtilisin ratio of 500:1 are shown in fig 6.1. From these results it can be seen that following 5 min proteolysis the PHCS subunit (49K) is the major band, but faint products are seen with molecular weights of 42.5K, 40K, 34K and 32K. From the results of the 30 min digest (track 12), it can be seen that the primary cleavage product is of molecular weight 34K, with a major secondary product at 32K. One faint band (<20K) is also apparent in 30 min digests.

c) Trypsin: The results of a limited time course digest using a PHCS (subunit):trypsin ratio of 100:1 are shown in fig 6.2. From these results it can be seen that following 2 min proteolysis, PHCS (49K) is cleaved to fragments of molecular weights 34K, 32.5K, 27K and 26K. After 30 min of digestion the native subunit (49K) and the band at 34K have disappeared leaving only major bands at 32.5K and 26K. Following 2h digestion the PHCS was digested to much smaller molecular weight products.

6.2 Peptide mapping of mAbs to proteolytic fragments of PHCS

Proteolysis of PHCS was repeated as in

Fig 6.2 Time dependent proteolysis of PHCS by trypsin
shown on an 8-12% gradient SDS PAGE gel



Tracks 1-6 PHCS fragments produced by limited proteolysis using trypsin: Time course: 10s (track 1), 2 min (track 2), 5 min (track 3), 10 min (track 4), 30 min (track 5), 2h (track 6).

Track 7. Undigested PHCS

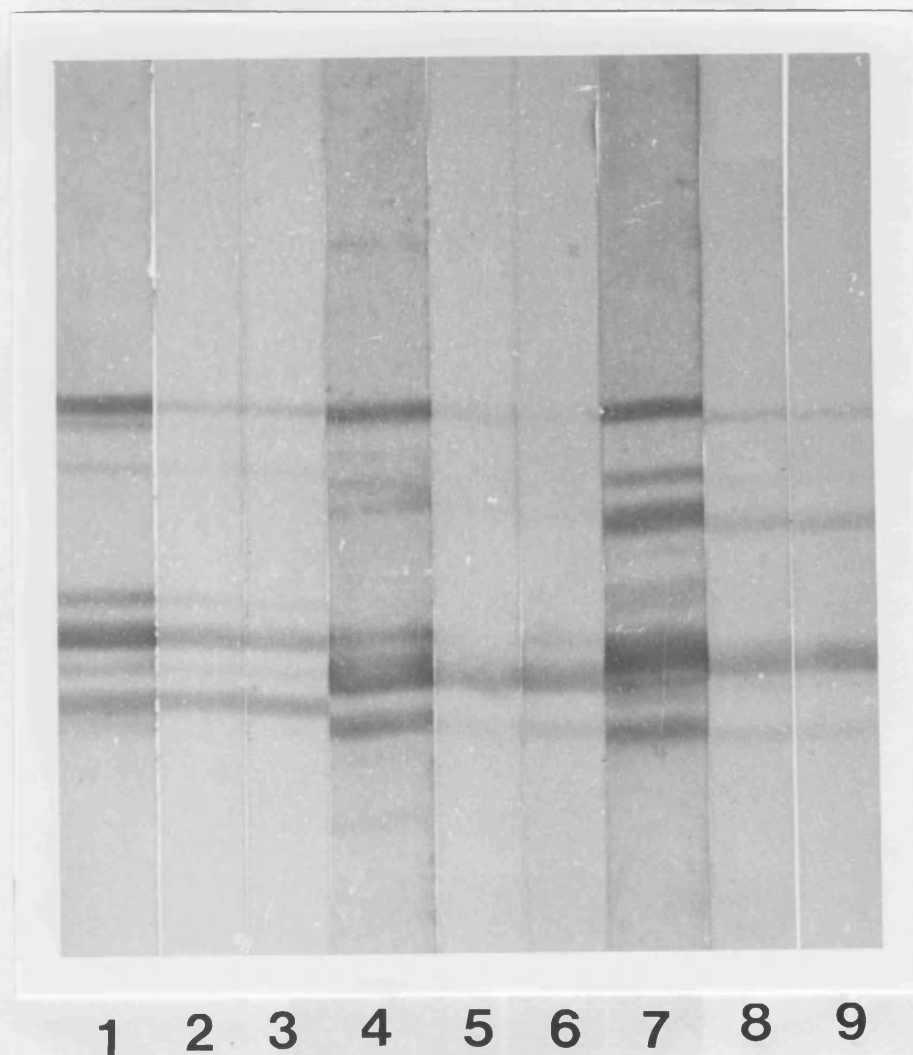
Track 8 Molecular weight markers (as for fig 6.1).

section 6.1, but single digestion times were chosen which gave the primary cleavage products found by Bloxham et al, 1982. Proteolysis was performed using chymotrypsin for 10 min, subtilisin for 45 min, or trypsin for 20 min. Following proteolysis, samples were run on 8-12% Laemmli SDS PAGE gels. Electrophoretic transfer of proteins from PAGE gels to nitrocellulose was performed essentially as in section 3.25, except that the transfer buffer was 25mM phosphate buffer, pH6.5, and transfer was carried out at 10V/0.24A for 16h. Immunoblotting of nitrocellulose-bound fragments of PHCS was performed as in section 3.29, using mAbs B and C, and mouse anti-PHCS serum diluted 1/100 in 1% casein/PBS.

For each experiment performed, 3 gel tracks, (molecular weight markers, undigested PHCS and fragmented PHCS, respectively) were stained, as described in section 3.27, but were not transferred to nitrocellulose. 3 similar tracks following transfer to nitrocellulose were stained with amido black, but were not immunoblotted. In this way it was shown that all the fragments of PHCS produced by chymotrypsin, trypsin and subtilisin, with molecular weights above 27K but not below this, were successfully transferred to nitrocellulose. Similarly, it was shown that mouse anti-PHCS serum was reactive with all the bands transferred to nitrocellulose.

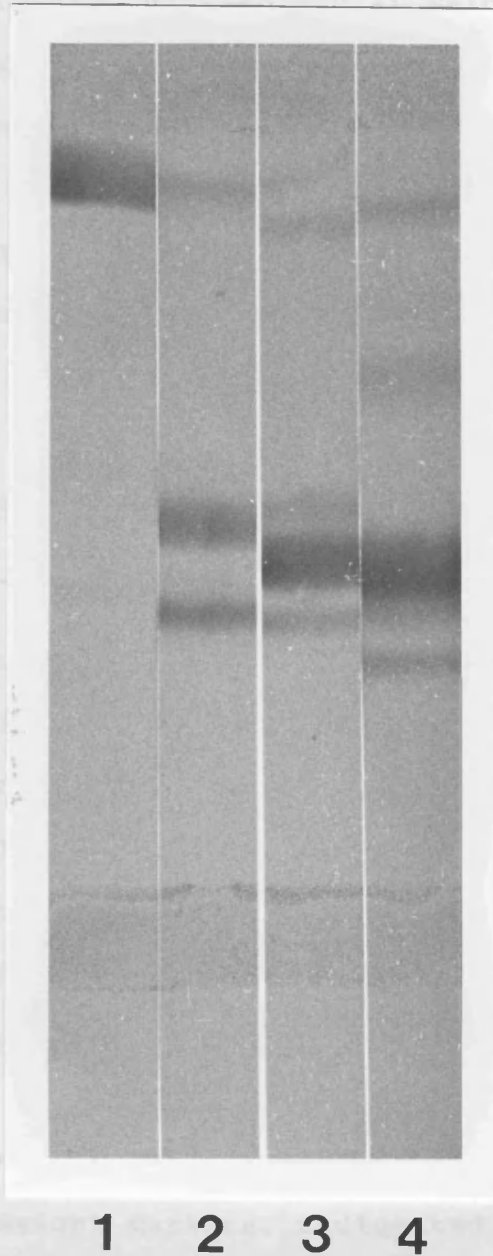
Results from the immunoblotting of proteolytic fragments of PHCS with mAbs B, C and mouse anti-PHCS serum are shown in figs 6.3 and 6.4.

Fig. 6.3 Proteolytic fragments of PHCS after immunoblotting with mAbs B,C and mouse anti-PHCS serum



Tracks 1-3; PHCS fragments following proteolysis by
chymotrypsin
Tracks 4-6; PHCS fragments following proteolysis by
trypsin
Tracks 7-9; PHCS fragments following proteolysis by
subtilisin
Tracks 1, 4 and 7; pattern shown following immunoblotting
with anti-PHCS serum
Tracks 2, 5 and 8; pattern shown following immunoblotting
with mAb B
Tracks 3, 6 and 9; pattern shown following immunoblotting
with mAb C

Fig. 6.4 Proteolytic fragments of PHCS after immunoblotting with mAb B



Track 1; undigested PHCS

Track 2; PHCS following proteolysis by chymotrypsin

Track 3; PHCS following proteolysis by trypsin

Track 4; PHCS following proteolysis by subtilisin

These results show that the major proteolytic fragments of PHCS, generated by a 10 min digestion with chymotrypsin, have molecular weights of 36K, 34K and 31K. Trypsin (20 min digest) produced major fragments of 34K, 33K and 31K, and subtilisin (45 min digest) gave strong bands of 39K, 33K and 31K.

The two mAbs (B and C) and the polyclonal anti-PHCS serum show strong reactivity with all these major bands.

6.3 Further peptide mapping of mAbs to a tryptic digest of PHCS

Proteolysis using trypsin was performed as in section 3.30, and samples prepared for SDS PAGE as in section 3.24. PAGE was performed using an 8-25% gradient gel in the Tris/Bicine system (section 3.23ii). As in section 6.2, 3 gel tracks (molecular weight markers, undigested PHCS and fragmented PHCS, respectively) were stained using coomassie blue, and the remaining tracks were transferred to nitrocellulose by the method described in section 3.25. Following transfer, 3 tracks (molecular weight markers, undigested PHCS and fragmented PHCS, respectively) were stained with amido black by the method described in section 3.28. The remaining tracks were immunoblotted (method 3.29) with mAbs B, C and E, normal mouse serum diluted 1/100 and mouse anti-PHCS serum diluted 1/100. For mAb E, 4%(w/v) PEG was included to aid complex formation during its incubation with a nitrocellulose strip containing digest

bands.

A graph of $\log_{10}\%T$ versus $\log MWt$ was plotted for the molecular weight standards, with data obtained from the stained gel (fig 6.5).

$\log_{10}\%T$ values were calculated from the distances travelled throughout the gel, for each fragment produced following proteolysis, and the corresponding molecular weights were read off the graph (fig 6.5). These results are summarized in table 6.1. The intensity of staining of each band in the gel by coomassie blue, as a measure of protein quantity, was visually rated on a scale of 1-6. The most intense band was assigned a value of 6 and the least intense a value of 1. These data are included in table 6.1.

The reactivities of antibodies following immunoblotting of PHCS fragments is shown in fig 6.6 and a summary of the reactivity of antibodies with fragments is included in table 6.1.

These results show that the major proteolytic fragments of PHCS, after a 20 min digestion by trypsin, have molecular weights of 40K, 33.0-35.5K, 23.5K, 13.5K and 9.5K. Polyclonal anti-PHCS serum shows a strong reactivity towards all these major bands except that of molecular weight 13.5K. MAbs B and C show reactivity with major bands at 40.0K, 33.0-35.5K and 9.5K, but not with bands at 23.5K or 13.5K. MAb E shows reactivity with major bands at 40.0K, 33.0-35.5K and 23.5K.

Fig 6.5 Graph of $\log_{10} \text{MWt}$ versus $\log \%T$ drawn for the molecular weight standards shown in fig 6.9.

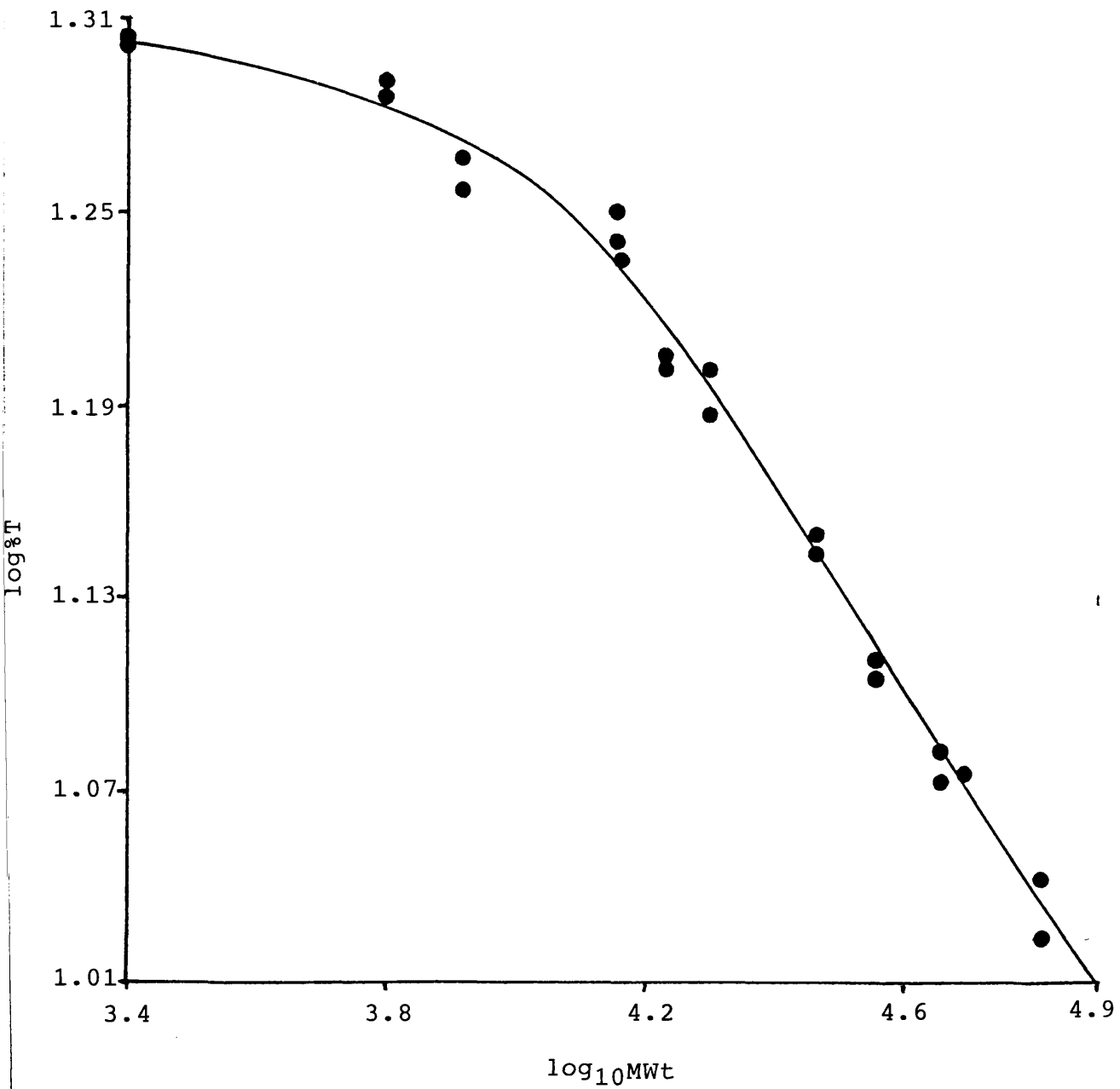


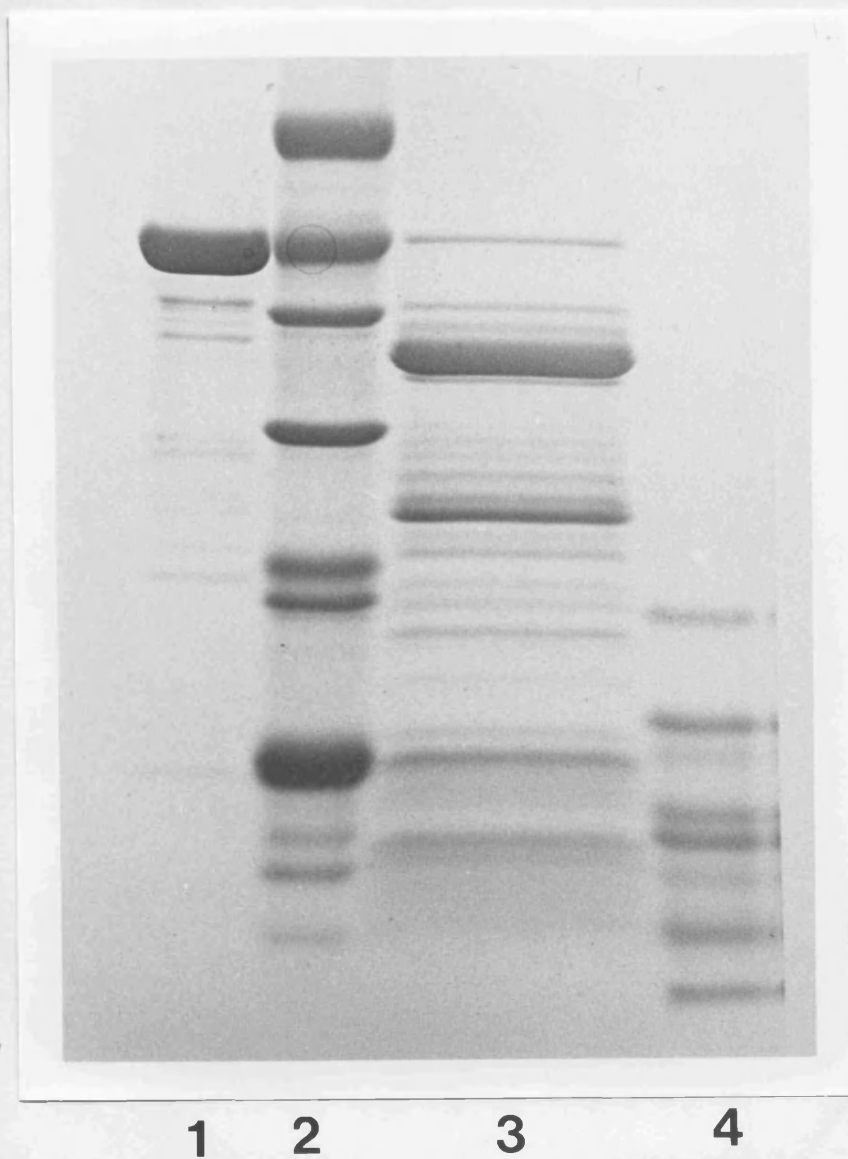
Table 6.1 Summary of the reactivity of the mAbs with tryptic fragments of PHCS

Fragment	MWt (K)	Band Intensity	Reactivity of Antibodies			
			mAbB	mAbC	mAbE	anti-PHCS serum
a	49.0	3	+	+	+	+
b	40.0	3	+	+	+	+
c	37.5	2	+	+	-	+
d	33.0-35.5	6	+	+	+	+
e	32.5	2	?	?	-	?
f	33.5	1	-	-	-	-
g	28.0	1	-	-	-	-
h	27.0	1	-	-	-	-
i	25.5	2	-	-	-	+
j	24.0	2	-	-	-	+
k	23.5	5	-	-	+	+
l	22.5	1	-	-	-	+
m	21.0	2	?	+	-	+
n	20.0	1	-	-	-	-
o	19.0	1	+	+	-	+
p	18.0	2	-	-	-	+
q	16.0	1	-	-	-	-
r	14.5	2	-	-	-	-
s	13.5	4	-	-	-	-
t	11.0-12.5	1	-	-	-	-
u	9.5	3	+	+	-	+
v	8.5	1	-	-	-	-
w	7.5	1	-	-	-	-
x	6.5	1	-	-	-	-
y	5.5	1	-	-	-	-

The intensity of the bands were assigned according to section 6.3.

Reactivity of antibodies is assigned on a positive (+) or negative (-) basis. Where a ? is assigned, the reactivity was uncertain.

Fig.6.6a 20 min proteolysis of PHCS by trypsin shown on an 8-20% gradient PAGE gel



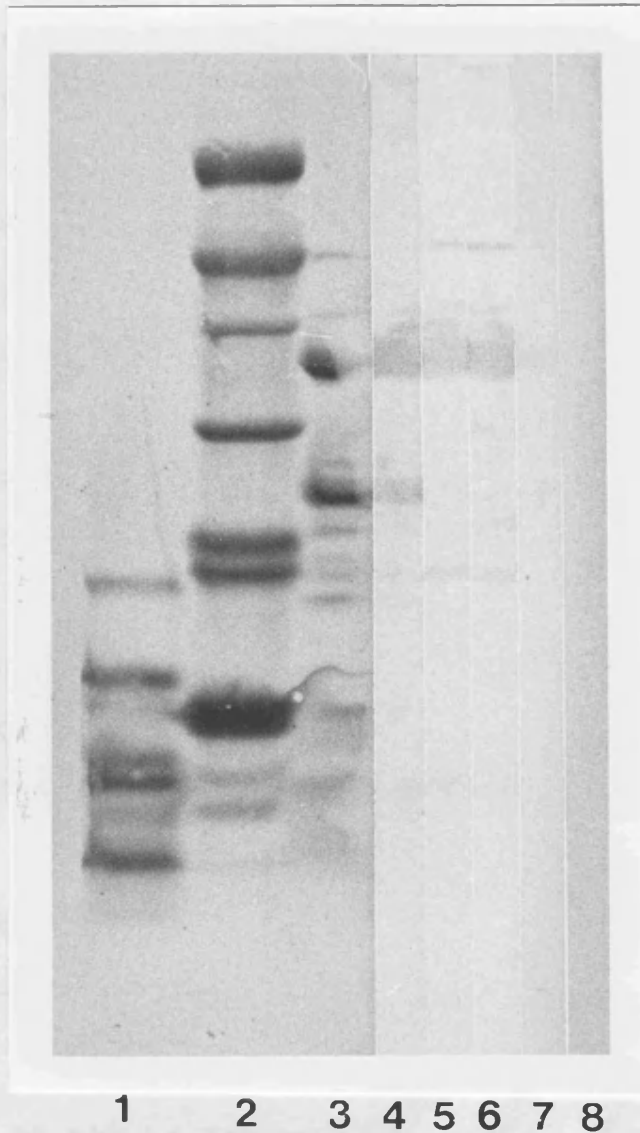
Track 1; undigested PHCS

Track 2; standards: 66K,45K,36K,29K,and 20K,(as per fig. 6.1),plus lactalbumin (14.2K)

Track 3; PHCS fragments produced following proteolysis by trypsin for 20 min

Track 4; standards: myoglobin (17K),myoglobin I+II (14.4K), myo. I (8.2K),myo. II (6.2K),and myo. III (2.5K)

Fig 6.6b Tryptic fragments of PHCS after immunoblotting
with mAbs B,C, and mouse anti-PHCS serum



Tracks 1,2 and 3; amido black staining of protein
 following electrophoretic transfer to
 nitrocellulose

Track 4; immunoblot pattern shown by anti-PHCS serum

Track 5; immunoblot pattern shown by mAb B

Track 6; immunoblot pattern shown by mAb C

Track 7; immunoblot pattern shown by mAb E

Track 8; immunoblot pattern shown by normal serum

Track 1; standards as per track 4, fig. 6.6a

Track 2; standards as per track 2, fig. 6.6a

Tracks 3-8; PHCS fragments following proteolysis by
 trypsin

6.4 Cleavage of asp-pro bonds by dilute acid

Cleavage of PHCS at asp-pro bonds was performed as described in section 3.31. Initially, a time course was performed covering the times 10 min, 20 min, 30 min, 1h, 2h and 4h. The products were run on a 8-20% Laemmli PAGE gel which included 6M Urea (fig 6.7). From tracks 2-9, it can be seen that the digest progresses with time over a period of up to 4h. After 10 min and 20 min, larger molecular weight fragments (>27K) predominate. As the reaction proceeds, up to 4h, only small molecular weight fragments (<17K) remain. Two time points were chosen for future use, a 30 min digest and a 90 min digest. From fig 6.7 it can be seen that these two times cover the whole range of fragments produced.

6.5 Peptide mapping of mAbs to acid cleaved fragments of PHCS

PHCS was cleaved at asp-pro bonds for 30 min and 90 min as described in section 3.31. The products were run on an 8-25% tris-bicine gel by the method described in section 3.23ii. Electrophoretic transfer of fragments to nitrocellulose, and immunoblotting, were performed as described in section 3.25 and 3.29.

A graph of $\log_{10} \%T$ versus $\log MWt$ (fig 6.8) was plotted for the molecular weight markers, with data obtained from the gel (fig 6.9). Figure 6.8 also shows that it was possible to transfer to

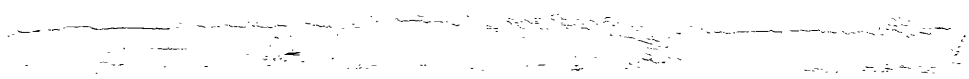
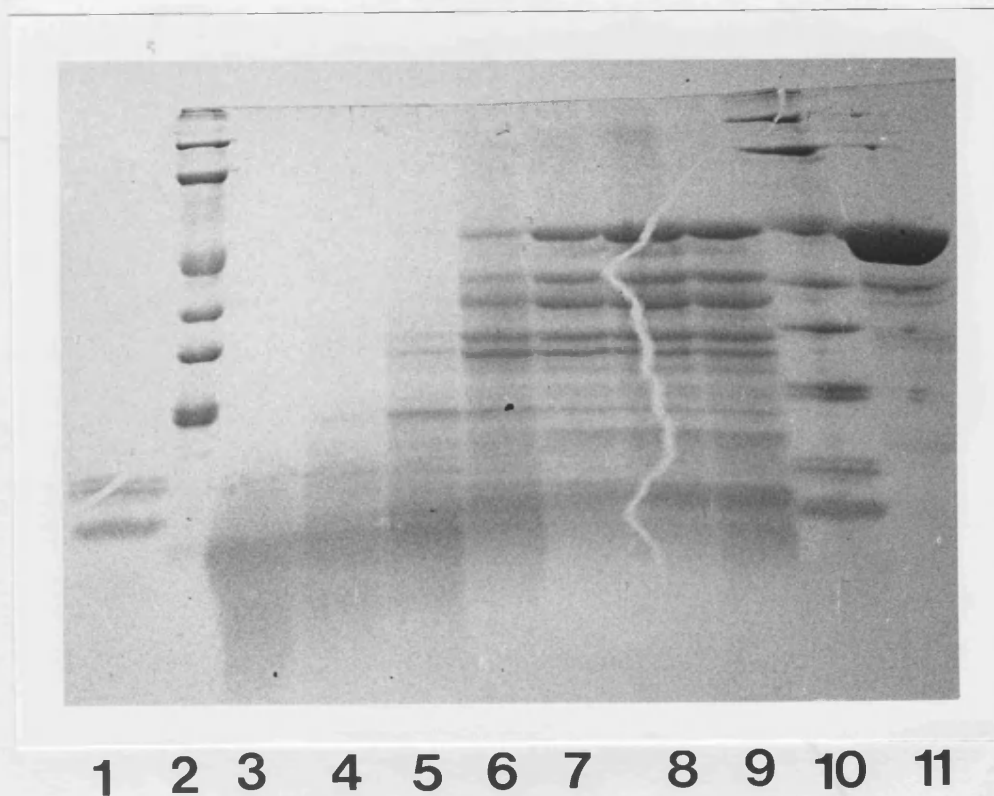


Fig.6.7 Time dependent cleavage of PHCS at asp-pro bonds by dilute acid, shown on an 8-20%, 6M urea, Laemmli PAGE gel



Track 11; undigested PHCS

Track 10; standards: 116K, 66K, 45K, 36K, 29K, 20K, 17K, 14.4K, 8.2K, and 6.2K

Tracks 9-3; Time course mild acid digest of PHCS (10 sec, 10 min, 20 min, 30 min, 1h, 2h, and 4h respectively)

Track 2; standards: 116K, 66K, 45K, 36K, 29K, and 20K

Track 1; standards: 17K, 14.4K, 8.2K, and 6.2K

The molecular weight standards used were the same as those used in fig. 6.6a.

Fig 6.8 Graph of $\log_{10} \text{MWt}$ versus $\log \%T$ drawn for the molecular weight standards shown in fig 6.6

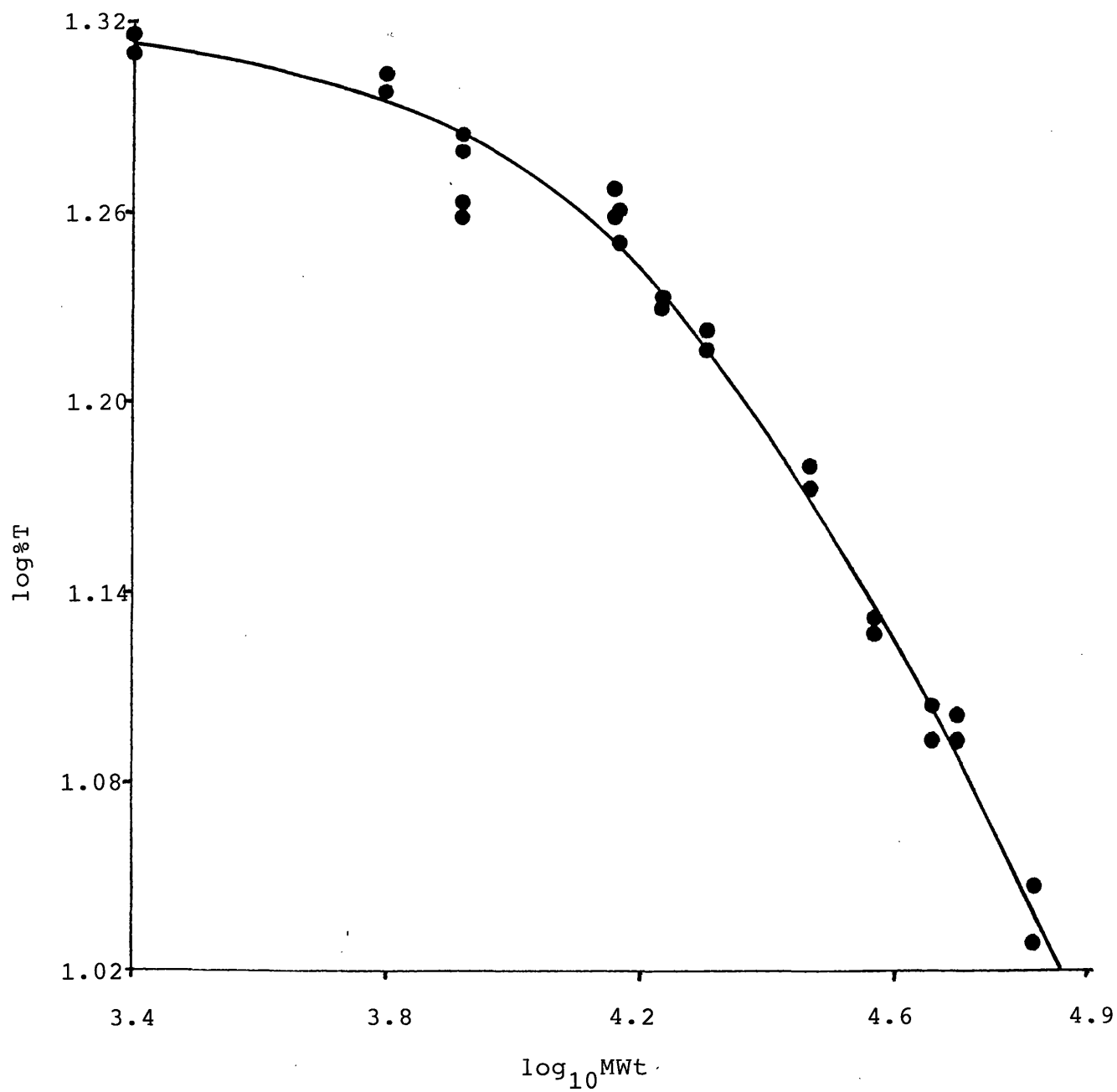


Fig 6.9 8-25% SDS PAGE gel, and nitrocellulose sheet,
showing PHCS fragments cleaved at asp-pro bonds



Tracks 1-5 SDS PAGE performed as per text 6.5.

Tracks 6-7 nitrocellulose following protein transfer,
performed as per text 6.5.

Track 1 undigested PHCS

Tracks 2 and 6 molecular weight standards; myoglobin (17K), myoglobin I and II (14.4K), myo I (8.2K), myo II (6.2K), myo III (2.5K)

Tracks 3 and 9 90 min cleavage at asp-pro bonds

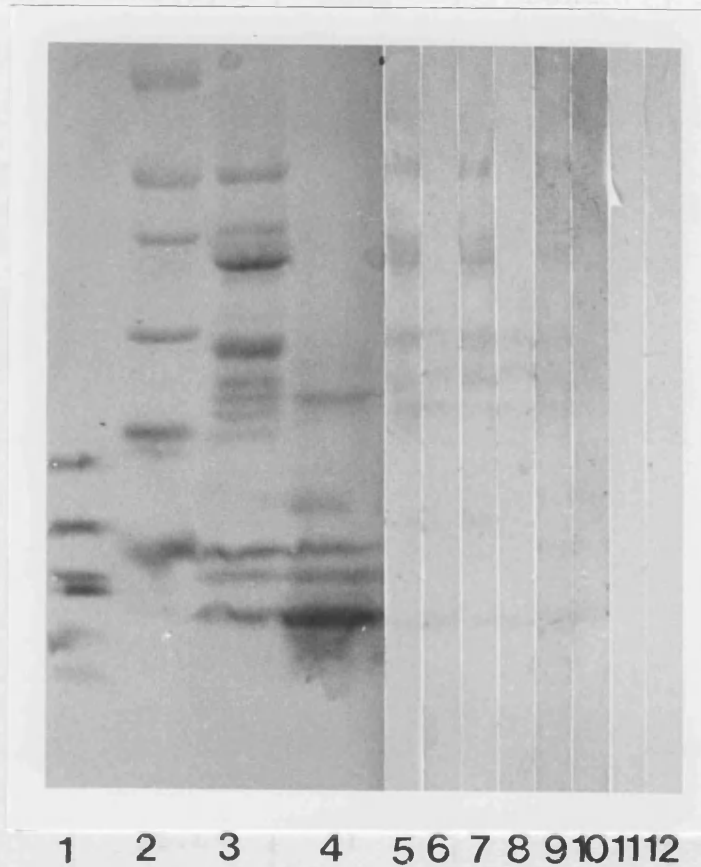
Tracks 4 and 7 molecular weight standards; BSA (66K), ovalbumin (45K), glyc-3-P-DH (36K), carbonic anhydrase (29K), trypsin inhibitor (20K), lactalbumin (14.2K).

Tracks 5 and 8 30 min cleavage at asp-pro bonds

nitrocellulose all bands following digestion. The results from the immunoblots performed, using mAbs B and C, normal mouse serum and mouse anti-PHCS serum are shown in fig 6.10. The data are summarized in table 6.2. The intensity of staining of each band in the gel by coomassie blue, as a measure of protein quantity, was visually rated on a scale of 1-6, the most intense band being assigned a value of 6, and the least intense a value of 1.

These results show the reactivity of mAbs B and C with acid cleaved PHCS are identical. Both mAbs react with products of molecular weights 6.5K-8.5K, 13.5K, 21.0K, 23.5K, 27.0K-27.5K, 33.5K, 33.5K-36.0K and 37.0K.

Fig 6.10 PHCS fragmented at asp-pro bonds, after immunoblotting with mAbs B, and C, normal mouse serum, and anti-PHCS serum



Tracks 1-4; amido black staining of protein following electrophoretic transfer to nitrocellulose
 Track 1; standards: 17K, 14.4K, 8.2K, 6.2K, and 2.5K, (as per fig. 6.6a)
 Track 2; standards: 66K, 45K, 36K, 29K, 20K, and 14.2K, (as per fig. 6.6a)
 Tracks 3, 5, 7, 9, and 11; 30 min mild acid hydrolysate of PHCS
 Tracks 4, 6, 8, 10, and 12; 90 min mild acid hydrolysate of PHCS

Immunoblots performed with mAb B (tracks 5 and 6), mAb C (tracks 7 and 8), anti-PHCS serum (tracks 9 and 10), and normal mouse serum (tracks 11 and 12)

Table 6.2 Summary of the reactivity of mAbs with PHCS fragments, produced by mild acid hydrolysis, defined by immunoblotting

Fragment	MWt(K)	Band Intensity 30 min digest	Band Intensity 90 min digest	Reactivity of antibodies		
				mAbB	mAbC	anti-PHCS serum
a	49.0	4	-	+	+	+
b	38.5-40.0	4	-	-	-	-
c	37.0	3	-	?	+	+
d	33.5-36.0	5	-	+	+	+
e	33.5	1	-	+	+	+
f	29.5	1	-	-	-	-
g	27.0-27.5	4	-	+	+	+
h	26.0	4	-	?	?	+
i	23.5	3	-	+	+	+
j	22.0	3	3	-	-	+
k	21.0	3	-	+	+	+
l	19.5	3	-	-	-	-
m	17.5	-	1	-	-	-
n	15.5	1	2	-	-	+
o	15.0	1	3	-	-	-
p	13.5	1	-	+	+	+
q	11.5-12.5	5	5	-	-	+
r	10.0-11.0	5	5	-	-	-
s	9.0	-	2	-	-	-
t	6.5-8.5	5	6	+	+	+
u	5.0	1	3	-	-	-
v	3.0-6.0	-	4	-	-	-

The intensity of the bands were assigned according to section 6.3.

Reactivity of antibodies is assigned on a positive (+), or negative (-) basis. Where a ? is assigned, the reactivity was uncertain.

6.6 MAB immunosorbent chromatography performed on PHCS fragmented at asp-pro bonds

MABs B and C were separately coupled to Sepharose 4B by the method described in section 3.32. With mAb B a coupling efficiency of 51% was achieved, leading to the production of a 1.5ml column with 2.55mg of coupled mAb. With mAb C a coupling efficiency of 60% was achieved leading to the production of a 1.5ml column with 3.00mg of coupled mAb.

Both columns were loaded with 300µg of PHCS hydrolysed (at asp-pro bonds) by treatment with mild acid for 90 min. MAB immunosorbent chromatography was performed as described in section 3.33. The bound peptides were eluted with 0.5M NH_4OH , pH10.8, freeze dried and run on 8-25% tris-bicine PAGE gels as described in section 3.23ii. The band patterns obtained from both the mAb B and mAb C columns were identical and an example is shown in fig 6.11. The resultant calibration curve for molecular weight markers is shown in fig 6.12. The results are summarized in table 6.3.

These results show that mAbs B and C are highly reactive with a 6.5-8.0K molecular weight fragment produced by acid hydrolysis of PHCS. A very weak reactivity may also be observed with a 13.0K molecular weight product.

Fig 6.11 8-25% SDS PAGE gel showing a 90 min mild acid hydrolysis of PHCS, and the fractions retained by the mAb C immunoaffinity column



Tracks 1,3 and 5. low molecular weight standards; myoglobin (17K), myoglobin I+II (14.4K), myo I (8.2K), myo II (6.2K) and myo III (2.5K)

Track 2. 90 min mild acid hydrolysis of PHCS

Track 4. The fraction of a 90 min mild hydrolysis of PHCS retained by mAb C immunoaffinity column.

Track 6. High molecular weight markers; as track 4, fig 6.9

Track 7. Undigested PHCS

Fig 6.12 Graph of $\log_{10} \text{MWt}$ versus $\log \%T$ drawn for the molecular weight standards shown in fig 6.11

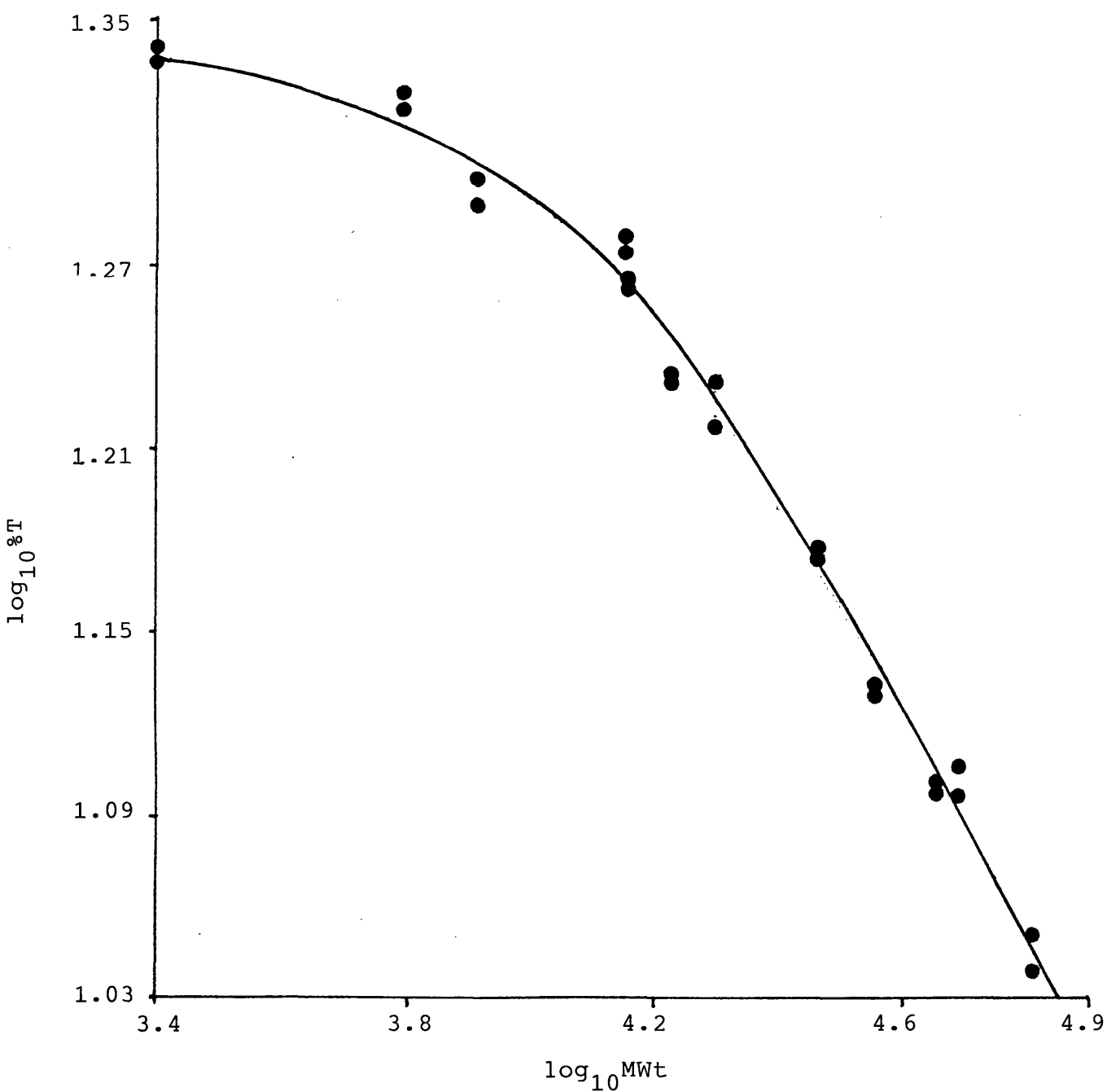


Table 6.3 Summary of the reactivity of mAbs with PHCS fragments produced by mild acid hydrolysis, defined by immunosorbent chromatography

Fragment	MWt (K)	Band Intensity	Reactivity of Antibodies	
			mAbB	mAbC
j	22.5	3	-	-
k	21.0	1	-	-
m	18.0	1	-	-
n	16.5	1	-	-
o	16.0	3	-	-
q	12.0-13.0	6	+	+
r	10.0-10.5	4	-	-
s	9.0	3	-	-
t	6.5-8.0	6	+++	+++
u,v	3.5-5.5	4	-	-

Results were from fig 6.11.

The intensity of staining of each band in the 90 min digest track were rated on a scale of 1-6. The most intense being assigned a value of 6, the least intense a value of 1.

Fragments were named according to their corresponding name in table 6.2.

6.7 Prediction of the products formed by cleavage of PHCS at asp-pro bonds

From the amino acid sequence of PHCS, fig 3, page 43, it can be seen that 4 asp-pro bonds occur. Using the known molecular weights of amino acids, the expected molecular weight of each product of a cleavage at asp-pro bonds was calculated. A complete and specific hydrolysis at asp-pro bonds would yield 5 products containing residues 1-59, 60-257, 258-327, 328-344 and 345-437. The expected products of a limited hydrolysis are shown in table 6.4.

Table 6.4 Theoretical products formed by cleavage of
PHCS at asp-pro bonds

Molecular weights of fragments containing amino acids

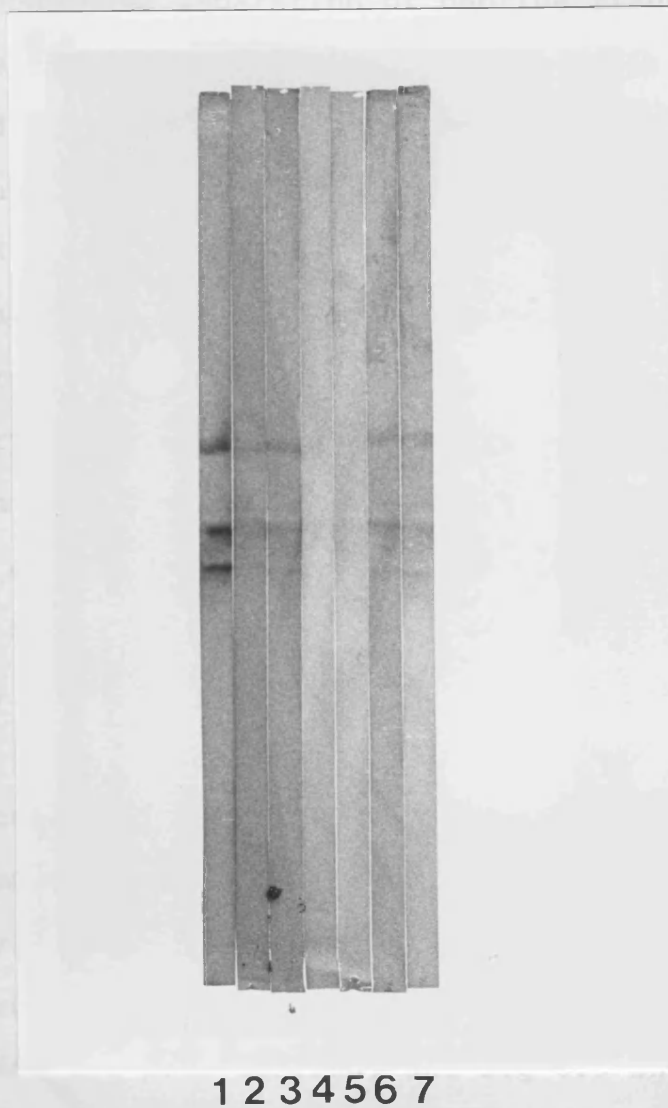
1-59	60-257	258-327	328-344	345-437
48969	48969	48969	48969	48969
38500	42500	42500	42500	42500
36400	38500	38500	38500	20300
28700	36400	36400	32000	12600
6500	32000	32000	20300	10500
	29900	29900	12600	
	28700	20300	9800	
	22200	9800	2100	
		7700		

6.8 Peptide mapping of mAbs to PHCS fragmented at asn-gly bonds

PHCS was cleaved at asn-gly bonds with hydroxylamine as described in section 3.31. The products were run on an 8-25% tris-bicine gel by the method described in section 3.23ii. Electrophoretic transfer of fragments to nitrocellulose and immunoblotting were performed as described in sections 3.25 and 3.29.

A graph of $\log_{10} \%T$ versus $\log M_w$ was plotted for the molecular weight markers. Only 3 bands were observed from a track of the digest; one corresponded to the native PHCS (49K), the remaining two bands had molecular weights of 27K and 18.5K. All three bands transferred well to nitrocellulose, and are shown in track 1 fig 6.13. From fig 6.13 it can be seen that mAbs B and C, and mouse anti-PHCS serum react with each of the three bands found following fragmentation of PHCS by hydroxylamine. Normal mouse serum shows no reactivity.

Fig 6.13 Immunoblots of PHCS fragmented at asn-gly bonds by hydroxylamine



Tracks 1-7; PHCS fragmented at asn-gly bonds as described in section 6.8, following transfer to nitrocellulose strips

Track 1; Resultant staining by amido black.

Track 2; Immunoblotting pattern given by mAb B

Track 3; Immunoblotting pattern given by mAb C

Tracks 4, 5; Immunoblotting pattern given by normal mouse serum

Tracks 6,7; Immunoblotting pattern given by mouse anti-PHCS serum.

6.9 Discussion

Initial experiments to effect the electrophoretic separation of peptide fragments involved SDS PAGE using the Laemmli system (section 6.1 and 6.2). It was found however that this did not give the required resolution of small fragments (1K-10K), even when larger gradients of 8-20% or 8-25% acrylamide were used. Adaptations were made similar to those of Hashimoto et al, 1983, in which the acrylamide:bisacrylamide ratio was changed from 37:1 in the Laemmli system to 20:1. This change did create a better resolution of the smaller molecular weight fragments. Inclusion of 6M urea in the gel system, however, appeared to be of no advantage.

The system finally adopted for use was a modification of the Tris-bicine system of Johnstone and Thorpe, 1982pp159-161, in which the acrylamide:bisacrylamide ratio was changed to 20:1. It can be seen from fig 6.6 that molecular weight bands from 2K to 66K are well defined, and fig 6.5 shows that molecular weight determinations within this range are readily performed.

The electrophoretic transfer of proteins from PAGE gels to nitrocellulose seemed to vary greatly in efficiency with the buffer used to perform it. 25mM phosphate buffer, pH6.5 was found to give a more efficient transfer than did 15.6mM Tris-120mM glycine, pH8.3, which is a popular transfer buffer, Gershoni and Palade, 1982. Both of these

buffers however gave very poor transfer of fragments with molecular weights between 2K and 20K, for both short (2h) and long (16h) transfer times. Passive transfer of proteins performed according to method 3.26 gave very good transfer (>50% after 36H); however, small molecular weight fragments had a tendency to diffuse laterally, so reducing the definition of bands obtained in PAGE.

The use of 2mM sodium acetate/5mM MOPS, 20% ethanol, pH7.5 in a transfer for 2h gave approximately 90% transfer of proteins in the molecular weight range 2K-20K, and approximately 70% transfer in the molecular weight range 20K-66K.

Previous studies of the proteolysis of PHCS by trypsin, chymotrypsin and subtilisin have shown that two predominant primary fragments are produced. The larger fragments, with molecular weights of 35K-38.5K, were shown to contain the amino terminal sequence of the native PHCS, while the small fragments, 9K-12K, were shown to contain the carboxy terminus (Wiegand, 1979, Bloxham, 1980). The primary cleavage sites of these proteolytic enzymes on PHCS have been established by Bloxham *et al*, 1981,1982. Further work by Lill *et al*, 1984, on the primary actions of these proteases on PHCS has verified these cleavage sites, which are summarized in table 6.5.

The proteolytic digest patterns shown in figs 6.1, 6.3 and 6.4 for chymotrypsin and subtilisin agree well with the data in table 6.5. The immunoblot

Table 6.5 Limited proteolysis of PHCS

Primary cleavage sites, determined by amino terminal sequence analysis (Lill et al, 1984; Bloxham et al, 1981, 1982). Molecular weights were calculated from the primary sequence.

Proteolytic Enzyme	Amino Acids Cleaved (Residue no.)	Primary Fragments	Molecular Weights (K)
Chymotrypsin	Leu-Arg	1-323	36K
	(323-324)	324-437	13K
Subtilisin	Ala-Leu	1-321	35.5K
	(321-322)	321-437	13.5K
	Val-Leu	1-322	36K
	(322-323)	323-437	13K
Trypsin	Arg-Val	1-313	35K
	(313-314)	314-437	14K
	Lys-Ala	1-366	41K
	(366-367)	367-437	8K

patterns observed show that mAbs B and C are reactive against the large 33-34K molecular weight bands of these digests. It is concluded from this that mAbs B and C bind to an epitope found between residues 1-321 of the native PHCS.

The proteolytic digest patterns shown in figs 6.2, 6.3 and 6.4 for trypsin agree well with the data in table 6.5. The immunoblot patterns observed show that mAbs B and C are reactive against the large 33-34K molecular weight bands of this digest. It is concluded from these data that mAbs B and C bind to an epitope found between residues 1-313 of the native PHCS.

However, due to the poor transfer of low molecular weight fragments these experiments do not prove that the mAbs cannot also bind to residues between 313-437 of PHCS.

In the further mapping studies performed in section 6.3, low molecular weight fragments were resolved well in PAGE, and transfer was very good to nitrocellulose. Using the Tris/bicine gel system, which gave the best resolution of small molecular weight bands, the primary fragments as per table 6.5, are well defined. For a cleavage at residues 313-314, the products at 33-35.5K, and 13K or 14.5K can be clearly seen. From the immunoblot results it is seen that mAbs B and C react well with the larger fragment, corresponding to residues 1-313. They show no reactivity however to the smaller fragment corresponding to

residues 313-437.

Lill et al, 1984, showed that for limited trypsinolysis the ratio of cleavage at residues 313-314 is twice that seen at residues 366-367. This explains the greater intensity of the product band at 33-35.5K over that seen at 40K. The small molecular fragment produced from a cleavage at 313-314 (14K) is less intense than its larger partner (33-35.5K), due to the likelihood of its also being cleaved at 366-367, producing further small products.

In this tryptic digest of PHCS a further major product is seen at 23.5K. Due to its intensity, and the fact that major primary cleavage sites are at 313-314 and 366-367, it must lie within the sequence 1-313. The immunoblotting data show that although the epitopes of mAbs B and C lie within the region 1-313 they are not present in this 23.5K subdivision of this region, but are however present in a 9.5K subdivision. Again due to its intensity the 9.5K fragment must lie within residues 1-313.

In a similar fashion it is seen that mAb E reacts with the major 33-35.5K product of a cleavage at 313-314, but does not react with the smaller 13.5 or 14.5 product of it. Therefore its epitope lies within the region 1-313. However mAb E reacts with the 23.5K cleavage product but not with the 9.5K product.

Peptide mapping studies, explained later, following cleavage of PHCS at asp-pro bonds, together with work performed using a synthetic peptide has

resolved the epitopes of mAbs B and C to lie within the region 258-313. This indicates that this region must be present on the 9.5K fragment, as mAbs B and C react with it.

Assuming that during the 20 min proteolysis, trypsin has been specific, with its cleavage sites being either at lysine or arginine, then only two sites of cleavage lie within the region 1-313 such that these two sized fragments can be produced and such that the 9.5K fragment contains the epitope present for mAbs B and C. These sites are lys-leu (206-207) and Arg-leu (229-230). This suggests that mAb E must bind to an epitope present within residues 1-230. Future verification of this may be performed by sequencing the fragments.

The data also show that mouse anti-PHCS serum does not react with the 13.5K or 14.5K fragment. This indicates that following immunization with the native antigen, antibodies are not readily produced to the regions of the protein between residues 314-437.

Previous studies have demonstrated that under acidic conditions asp-pro peptide bonds are cleaved more rapidly than any other peptide bonds normally present in proteins (Piszkiewicz, 1970). The present study uses the method of Rittenhouse and Marcus, 1984, to perform peptide mapping of PHCS following cleavage at asp-pro peptide bonds. Table 6.4 shows the molecular weights of the expected products of a limited acid hydrolysis of PHCS. Including the

native enzyme a total of 15 fragments would be expected. Results from table 6.2 show that hydrolysis of PHCS by mild acid produces a total of 22 fragments, showing that some cleavage other than at asp-pro bonds has occurred. This cleavage is likely to occur at an asn-pro bond occurring at residues 369-370.

The immunoblots following trypsinolysis have shown that mAbs B and C bind to epitopes between residues 1-313. From table 6.4 it can be seen that the molecular weights of fragments corresponding to residues 328-344 and 345-437 are 2.1K and 10.5K respectively. Results summarized in tables 6.2 and 6.3 show that mAbs B and C are not reactive with fragments r and v corresponding to these molecular weights. This shows that mAbs B and C do not react with an epitope between 328-437, corroborating the results found following trypsinolysis.

Following mild acid hydrolysis, the major band (j) of molecular weight 22K corresponding to the expected molecular weight of the fragment containing residues 60-257 is not reactive with mAbs B and C. For an epitope between these residues to be recognised no products below 22K should be reactive with mAbs B and C. However fragments of 21.0K, 13.5K and 6.5-8.5K are reactive with mAbs B and C. Thus the epitopes recognised by mAbs B and C must be present either between residues 1-59, or 258-327.

From table 6.3 it is seen that the molecular weights of fragments corresponding to residues 1-59

and 258-327 are 6.5K and 7.7K respectively. From the fragments observed (tables 6.2 and 6.3) it is likely that fragment t (6.5K-8.5K) corresponds to the 7.7K fragment expected for residues 258-327, and fragment v (3.0K-6.0K) corresponds to the 6.5K fragment expected for residues 1-59. As mAbs B and C react with only one of these fragments (6.5K-8.5K) it seems likely that their epitopes lie between residues 258-327. From table 6.4 it can be seen that only 4 fragments, excluding the native PHCS, are expected to contain residues 1-59, and only one of these (6.5K) has a molecular weight below 28K. However, table 6.2 shows that mAbs B and C react with fragments of molecular weights 27-27.5K, 23.5K, 21.0K, 13.5K and 6.5-8.5K. From table 6.4 it can be seen that 8 fragments, excluding native PHCS, are expected to contain residues 258-327, and 3 of these (20.3K, 9.8K and 7.7K) have molecular weights below 28K. This evidence also suggests that the epitopes recognised by mAbs B and C are between residues 258-327. Combining this with the results following trypsinolysis, the epitopes recognised by both mAbs B and C lie between residues 258-313.

Further work in our laboratory by Daham Ali has shown that mAbs B and C both recognise a synthetic peptide corresponding to residues 288-302, when assayed by direct ELISA. This work shows that the epitope recognised by both mAbs B and C does indeed lie between residues 288-302.

Following cleavage at asn-gly bonds (section 6.8), only 2 products were observed with molecular

weights of 28.0K and 18.5K. Only 1 asn-gly bond occurs in PHCS, at residues 267-268. The expected molecular weights of fragments produced following cleavage of this bond are 30K and 19K, correlating well with the observed products. The binding of mAbs to both fragments might suggest that part of the epitope exists in each fragment. This may mean that the epitopes recognised by mAbs B and C both contain part of the sequence between residues 258-267 plus part of the sequence 288-302.

CHAPTER SEVEN COMPUTER PREDICTIONS OF THE EPITOPES
PRESENT ON PHCS

	Page
7.1 Introduction.	172
7.2 Prediction of the epitopes of PHCS using hydrophilicity data.	172
7.3 Prediction of the epitopes of PHCS using 'Composite surface profile' data.	175
7.4 Prediction of the epitopes of PHCS using atomic mobility values.	177
7.5 Prediction of the epitopes of PHCS using 'protrusion indices'.	178
7.6 Prediction of the surface residues of PHCS existing in continuous regions.	179
7.7 Discussion.	183

7.1 Introduction

This chapter describes the methods used to predict the antigenic sites present on PHCS. The rationale for the use of these methods has been discussed in sections 1.4-1.4.6.

These studies have been performed by other workers in collaboration with our group, and alongside the experimental approach performed by myself.

7.2 Prediction of the epitopes of PHCS using hydrophilicity data

This procedure was first described by Hopp and Woods, 1981.

From the solvent parameters of Levitt, 1976, each of the 20 amino acids commonly found in proteins is assigned a hydrophilicity value. The values used are shown in table 7.1. A hydrophilicity profile is achieved by repetitively averaging these values for overlapping sets of 6 residues along the chain and plotting them versus the residue number of the leading residue.

The hydrophilicity profile for PHCS was performed by S.A. Garforth, 1985. In this work he adapted a program written in Hewlett-Packard BASIC, for use on a BBC micro-computer. The profile so obtained is shown in fig 7.1.

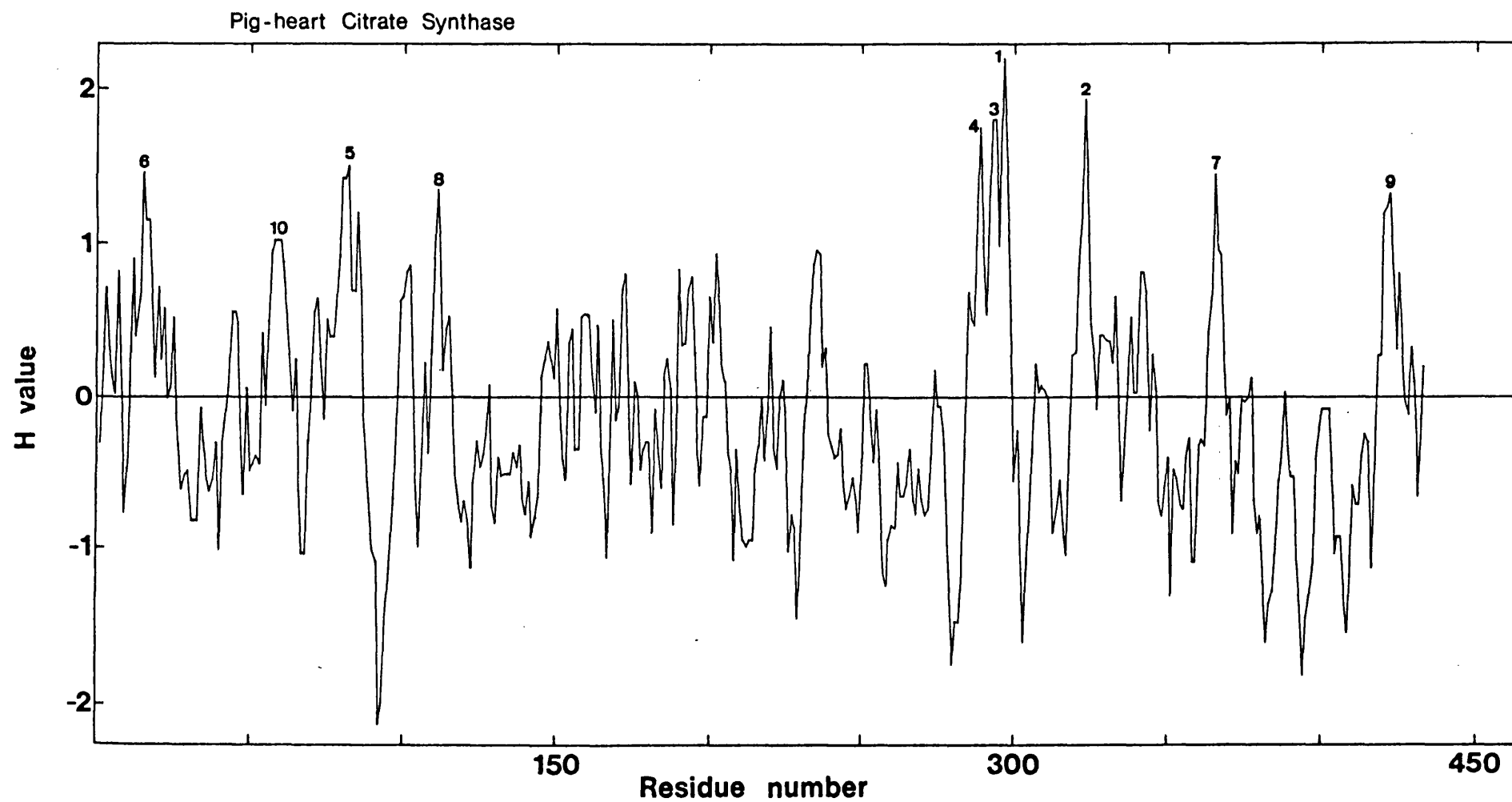
From the results shown in fig 7.1 it can be seen that the top five peaks of hydrophilicity occur at residues 297-302, 323-328, 293-299, 289-294

Table 7.1 Hydrophilicity values assigned to individual amino acids

<u>AMINO ACID</u>	<u>HYDROPHILICITY</u>
	<u>VALUE*</u>
Arginine	3.0
Aspartic acid	3.0
Glutamic acid	3.0
Lysine	3.0
Serine	0.3
Asparagine	0.3
Glutamine	0.2
Glycine	0.0
Proline	0.0
Threonine	-0.4
Alanine	-0.5
Histidine	-0.5
Cysteine	-1.0
Methionine	-1.3
Valine	-1.5
Isoleucine	-1.8
Leucine	-1.8
Tyrosine	-2.3
Phenylalanine	-2.5
Tryptophan	-3.4

* Based on the solvent parameter values of Levitt,
1976.

Fig 7.1 Hydrophilic profile



and 82-87. It should however be noted that according to Hopp and Woods only the highest peak of hydrophilicity was invariably found to correlate with antigenic sites in the model protein systems they used. For PHCS the highest peak of hydrophilicity occurs between residues 297-302.

7.3 Prediction of the epitopes of PHCS using 'Composite Surface Profile' data

This procedure was described by J.M.R. Parker 1986.

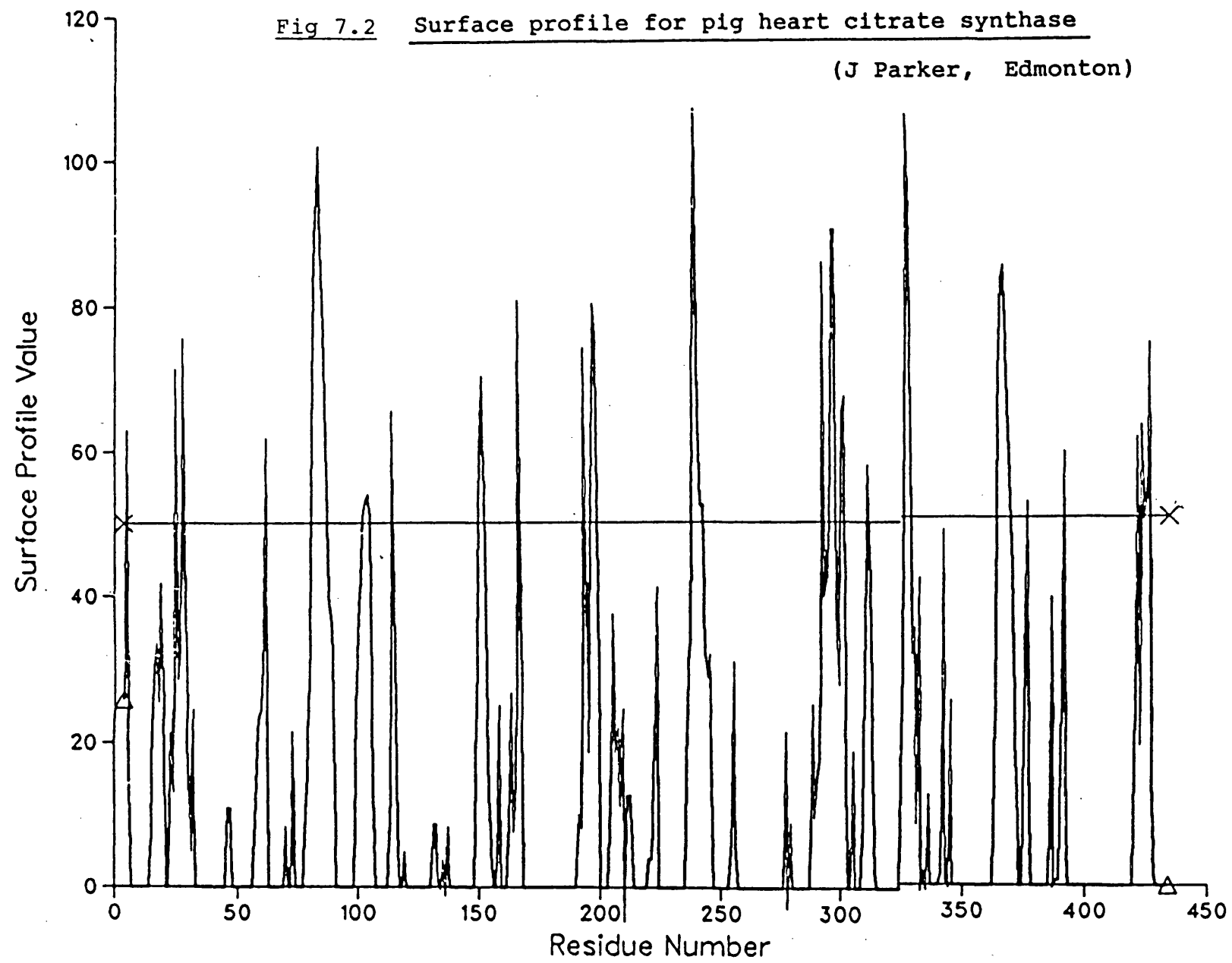
Each of the 20 amino acids commonly found in proteins was assigned a 'surface profile value'. The 'surface profile' values were arbitrarily assigned on a scale of 0-100, and are a composite of HPLC hydrophilicity indices, accessibility and flexibility values, as described in section 1.4.5. A composite surface profile is achieved by repetitively averaging these values for overlapping sets of 7 residues along the chain, and plotting them versus the fourth residue number of each set of 7.

The composite surface profile for PHCS was performed by J.M.R. Parker, and is shown in fig 7.2.

From the results shown in fig 7.2, it can be seen that the top five peaks of composite surface profile occur at residues 236-246, 325-332, 78-90, 288-302 and 363-372. Parker, personal communication, suggests that the top 10 peaks may be considered as possible antigenic sites.

Fig 7.2 Surface profile for pig heart citrate synthase

(J Parker, Edmonton)



7.4 Prediction of the epitopes of PHCS using atomic mobility values

Use of X-ray crystallography gives not only the precise atomic coordinates, but also atomic temperature factors (B values). The temperature factor represents the mean-square displacement of each atom, and when plotted against residue number gives a graphic image of the degree of mobility existing along the polypeptide chain, as described in section 1.4.4.

The B-values obtained for the atoms in PHCS were determined by Remington et al, 1982. The mobility profile is shown in fig 7.3.

From the results shown in fig 7.3, it can be seen that three major peaks of mobility occur at residues 81-85, 285-302 and 1-5.

7.5 Prediction of the epitopes of PHCS using protrusion indices

This procedure was first proposed by Thornton et al, 1986 and has been described in section 1.4.2. Protrusion indices have been calculated for PHCS from its X-ray crystallographic atomic coordinates. These PI values are calculated values for each amino acid of the PHCS and are a measure of the extent to which a residue protrudes out from the surface of the molecule.

The data used were provided by Dr.J.M. Thornton (Birbeck Coll, University of London), 1986.

Fig 7.3 Main-chain B-factors for citrate synthase

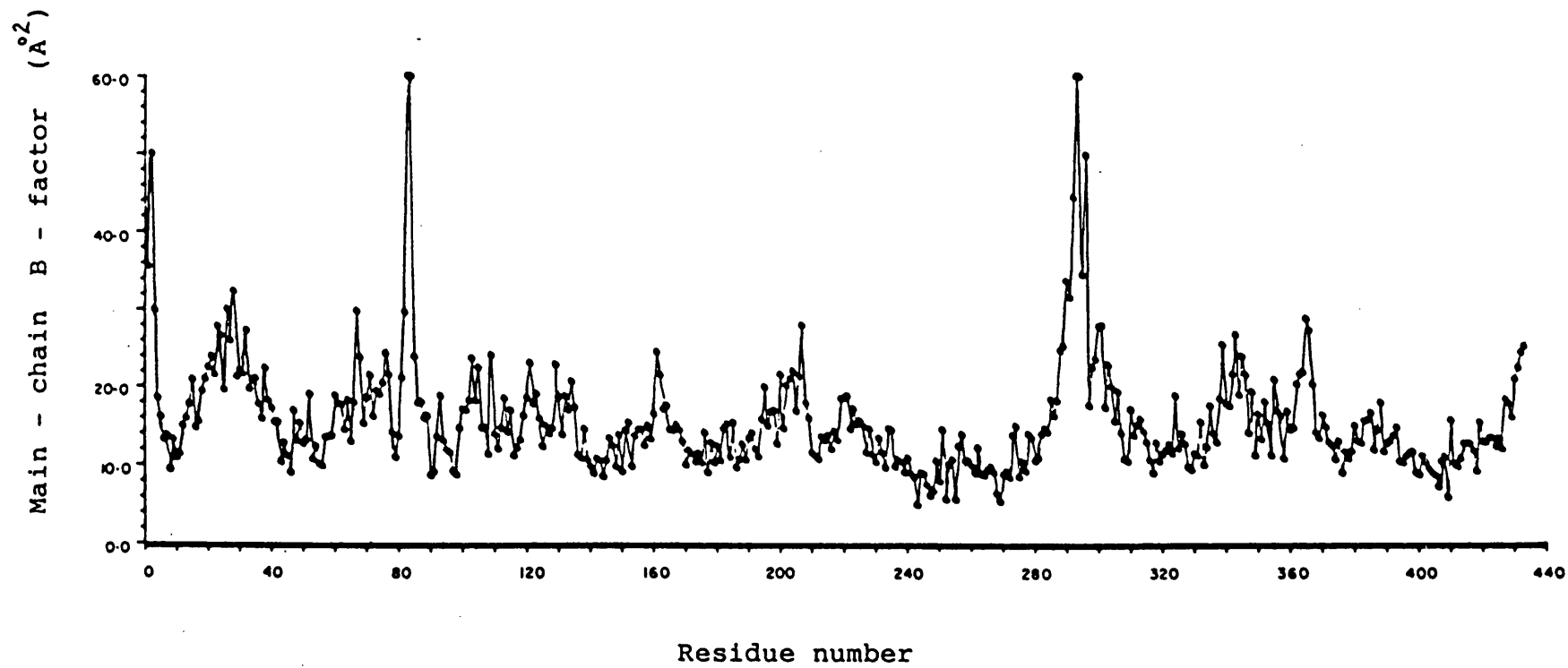


Fig 7.4 shows a protrusion index profile drawn by plotting the PI value of each amino acid against its residue number.

A further protrusion index profile was obtained by repetitively averaging PI values for overlapping sets of 6 residues along the chain and plotting them versus the residue number of the last residue in the set. This data is presented in fig 7.5.

From these data it can be seen that the most protrusive regions contain residues 421-437, 119-137, 14-42, 287-313, 154-166 and 191-202.

7.6 Prediction of the surface residues of PHCS existing in continuous regions

This work was first described by Barlow et al, 1984.

The method used involves centering a sphere of radius 6\AA on each surface atom of the protein, and calculating the proportion (F) of the centres of other surface atoms enclosed by the sphere which belong to residues local in the amino acid sequence. Surface atoms were defined as those with contact areas $>2\text{\AA}^2$. Local surface atoms were defined as those ± 3 residues distant in the sequence of the residues under consideration. Residues which contain no surface atoms were assigned a value of $F=0$.

The 'continuous surface' profile was performed by Dr. J.M. Thornton, 1986. These data are presented in fig 7.6. From these data, using a cut off at 50%

Fig 7.4 Protrusion index profile of PHCS

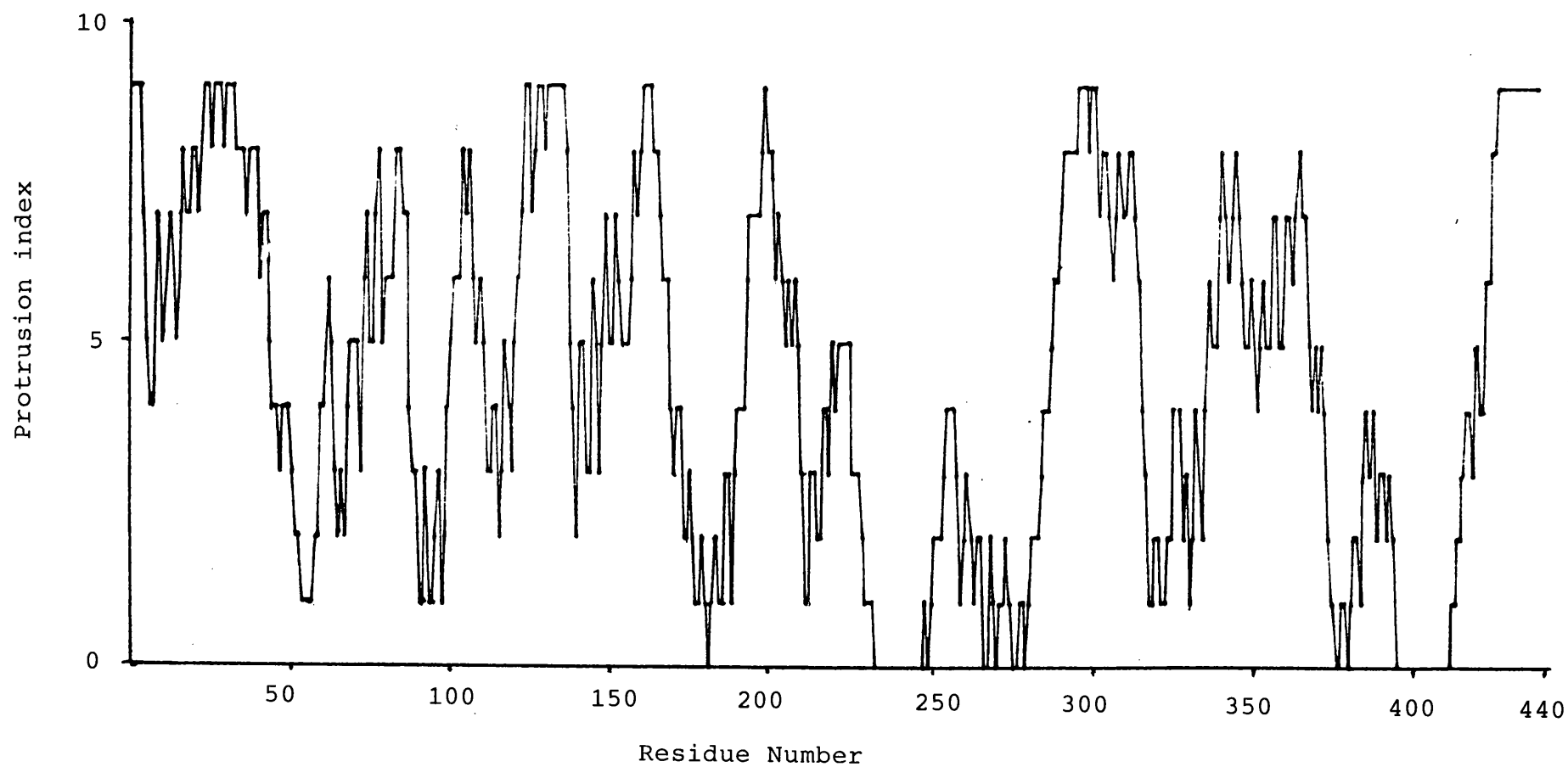


Fig 7.5 Averaged protrusion index profile of PHCS

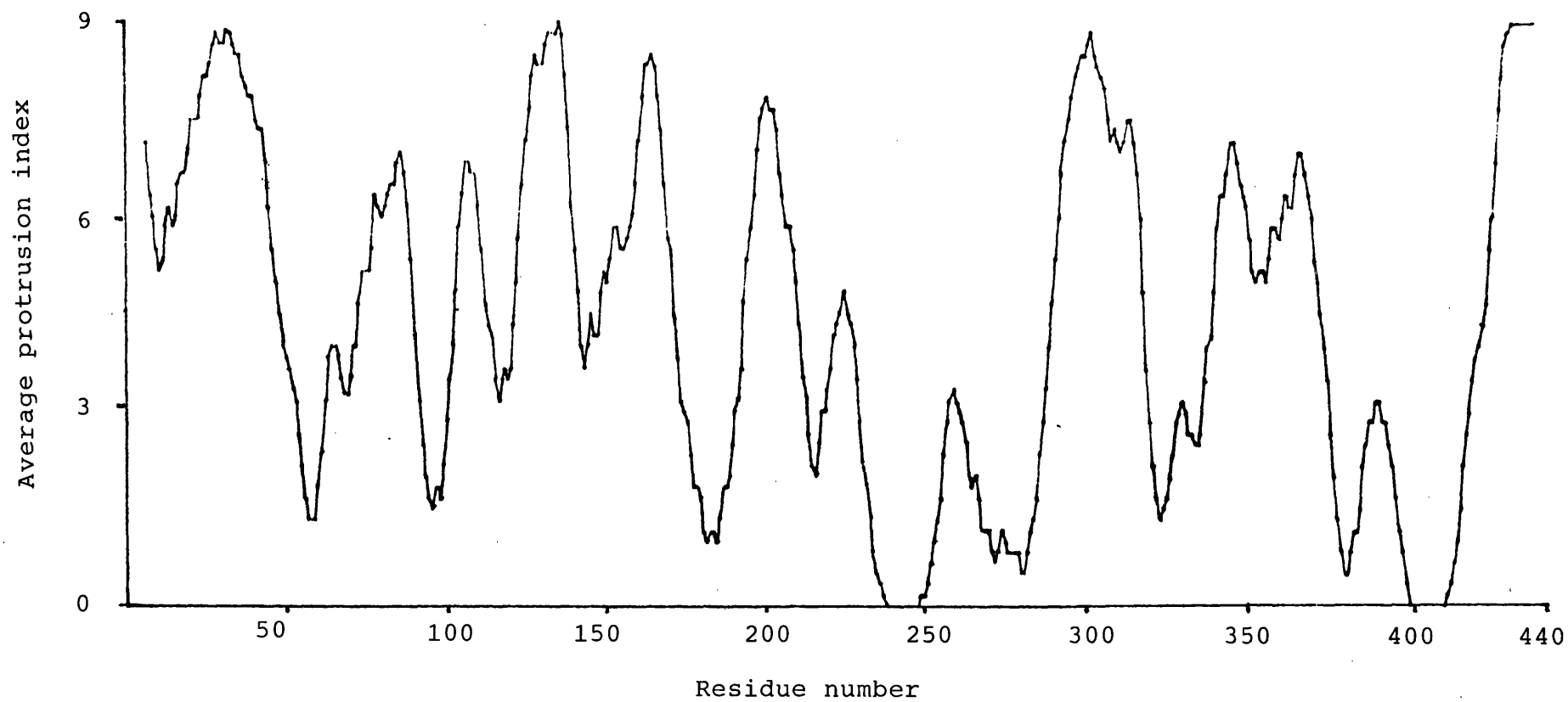
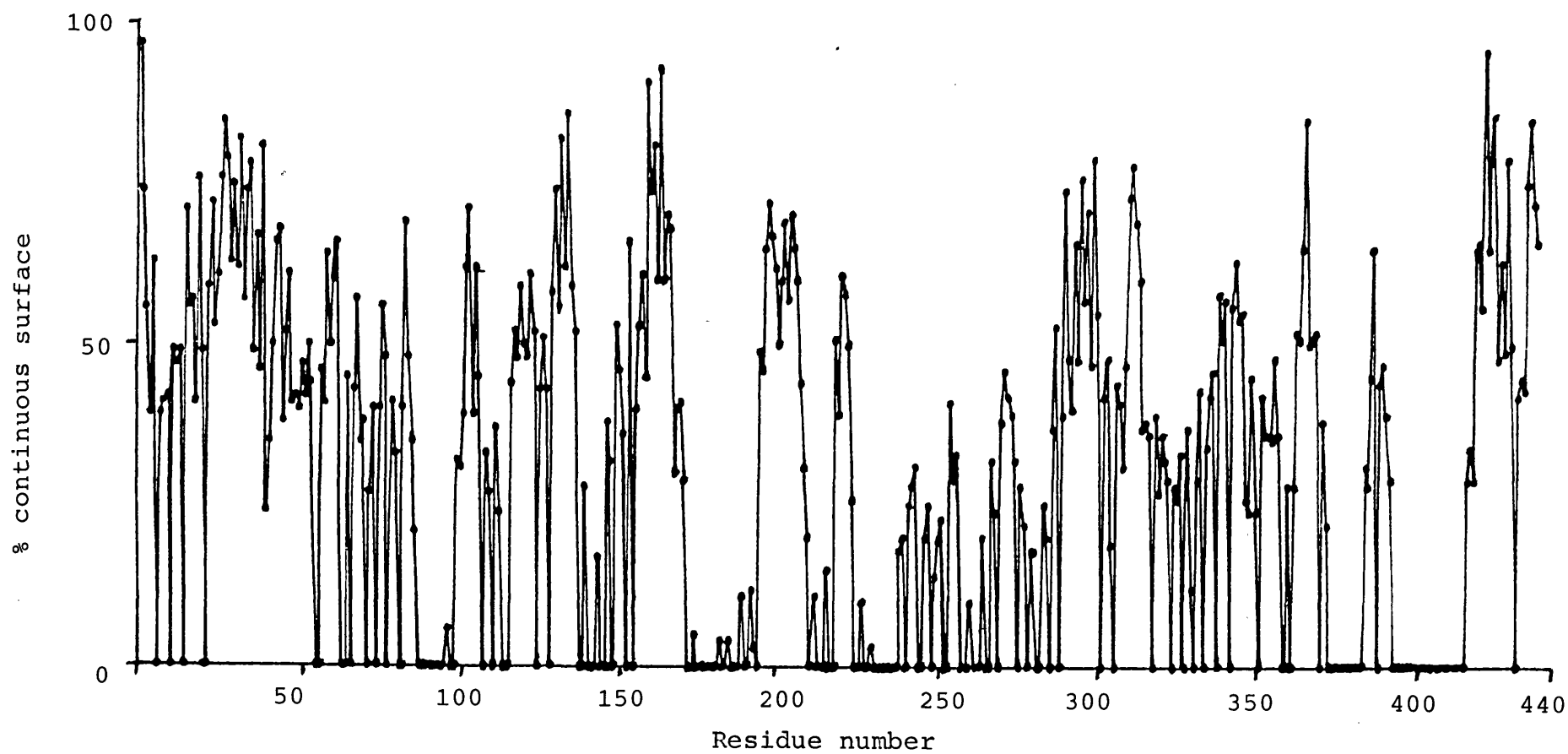


Fig 7.6 Histogram of % continuous surface (F) for residues in PHCS



'continuousness', it was predicted that the most likely continuous antigenic sites would contain residues 418-424, 201-206, 159-166, 129-136 and 25-35.

7.7 Discussion

In this chapter five different predictive approaches have been applied to compute the likely antigenic sites present on PHCS. All predictions were performed for the monomer of PHCS, with 437 residues. The first two approaches (hydrophilicity indices, and composite surface profile values), use empirical values for each amino acid which are then applied to the primary sequence data. The remaining approaches (mobility predictions, protrusion (indices) and continuous surface profile) rely on physical data obtained by X-ray crystallography.

The major antigenic sites predicted by each method are shown on table 7.2. Predictions made by methods 7.2, 7.3, 7.4 and 7.5 all suggest that a region contained within residues 288-302 is likely to be an antigenic site. Results from method 7.6 do not predict this region to contain a continuous epitope as residues 288, 290, 291, 292, 294, 298, 301 and 302 show less than 50% 'continuousness'. Therefore an epitope is strongly predicted to occur between residues 288-302, but it is likely to be partly discontinuous. Two further discontinuous epitopes (residues 81-87 and 323-328) are predicted by methods

Table 7.2 Summary of the predicted epitopes of PHCS

(A) <u>Hydrophilicity</u>	(1) 297-302)
	(2) 323-328
	(3) 293-299
	(4) 289-294
	(5) 82-87
(B) <u>Composite surface profile</u>	(1) 236-246
	(2) 325-332
	(3) 78-90
	(4) 288-302
	(5) 363-372
(C) <u>Mobility</u>	(1) 81-85
	(2) 285-302
	(3) 1-5
(D) <u>Protrusion</u>	(1) 421-437
	(2) 119-137
	(3) 14-42
	(4) 287-313
	(5) 154-166
	(6) 191-202
(E) <u>Surface Continuogram</u>	(1) 418-424
	(2) 201-206
	(3) 159-166
	(4) 129-136
	(5) 25-35

7.2, 7.3, 7.4 and methods 7.2, 7.3, respectively.

Methods 7.5 and 7.6 predict continuous antigenic sites to exist at residues 159-166, 129-136 and 25-35. Methods 7.5 and 7.6 were however performed on the monomeric subunit of PHCS, thus some of the sites predicted may be obscured upon dimer formation.

CHAPTER EIGHT

CONCLUDING REMARKS

The first stage of this project (chapter 4) was the production of a library of 11 mAbs against PHCS. In this report the importance of multiple re-cloning of cells has been emphasized, to reduce the risk of overgrowth by non-antibody producing cells. In my work approximately 4-5% of growing cells after fusion were found to secrete antibody reactive with the antigen used for immunisation. This agrees with the work of Goding, 1980, whose fusion strategy was followed.

Of the mAbs raised 9 were classified as being of IgM type and 2 of being IgG type. This higher ratio of IgM to IgG secreting cells is generally reported in mAb work.

Investigation of a series of solid phase immunoassays (chapter 5) has shown how antigen presentation has a significant effect on the reactivity of mAbs. Of 12 different direct and solid phase immunoassays tested, a sandwich ELISA proved to be the most useful for detecting all of a heterogeneous group of mAbs. The superiority of this assay over the other systems used is thought to be due to its ability to present simultaneously a soluble antigen in a variety of orientations or conformations. This might be expected from the variety of specificities of anti-PHCS present in the coating polyvalent antibody preparation.

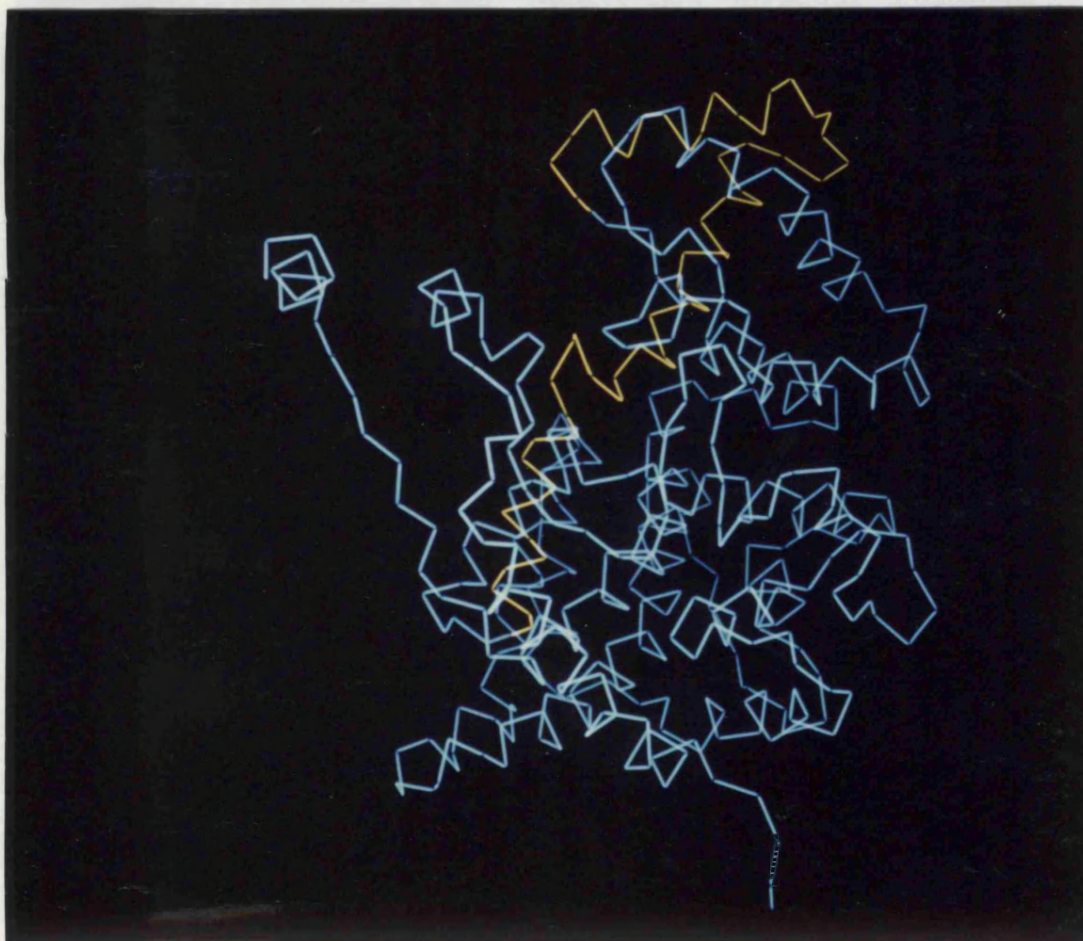
Sandwich ELISAs were developed in which the

antigen is presented in a 'closed' form (with oxaloacetate bound), and in an 'open' form (with no ligand present), see section 5.4. This work has shown that mAb E is able to bind equally well to PHCS in both its 'open' and 'closed' forms. However mAbs B and C bind much less well to PHCS in the 'closed' form than to it in its 'open' form. Wiegand et al, 1984, have shown that the conformation of the small domain (residues 275-380, fig.4), changes considerably between the different forms of the enzyme. However, the conformation of the large domain (residues 1-274 and 381-437) is fairly constant in all forms of the enzyme. Thus the reactivity of mAbs with the two forms of the enzyme suggests that mAbs B and C react between residues 275-380, and mAb E reacts with an epitope present either between residues 1-274 or 381-437.

Epitope mapping studies (chapter 6) performed on fragments of PHCS produced by enzymic and chemical cleavage have defined the regions recognised by mAbs B, C and E. MAb B and C recognise an epitope lying between residues 258-313, shown in fig.8.1, and mAb E recognises an epitope between residues 1-230. Further studies using a synthetic peptide have shown that mAbs B and C bind to epitopes between residues 288-302, shown in fig.8.2. Thus the epitopes recognised by mAbs B and C are on an exposed surface loop of PHCS.

This experimentally determined epitope within residues 288-302 is predicted to be antigenic using

Fig.8.1. Computer graphics image of PHCS highlighting the position of residues 258-313



This computer graphics image shows the backbone of the amino acid sequence of a monomer of PHCS. The position of residues 258-313 is highlighted in yellow, the remainder of the molecule being shown in blue.

Fig.8.2. Computer graphics image of PHCS highlighting the position of residues 288-302



This computer graphics image shows the backbone of the amino acid sequence of a monomer of PHCS. The position of residues 288-302, in an exposed loop, is highlighted in yellow, the remainder of the molecule being shown in blue. The two arrows (in black) show the position at which binding occurs with the second monomer.

hydrophilicity data (section 7.2), composite surface profile data (section 7.3), atomic mobility values (section 7.4), and protrusion indices (section 7.5). This epitope is however predicted to be partly discontinuous by a surface continuogram (section 7.6).

This work has successfully shown how the antigenic sites recognised by antibodies can be defined experimentally by:

a) Immunoassays designed to present an antigen in different set conformations.

and b) Epitope mapping studies performed on fragments of the antigen.

The simultaneous use of a predictive approach to determine antigenic sites has shown the value of four methods (sections 7.2-7.4) in their ability to predict epitopes.

In a continuation of this project, further experimental work is to be performed to delineate the epitopes recognised by mAbs A,D,E,F,G,H,K,L and M. These data will enable a more complete evaluation to be performed of the relative merits of the various methods discussed for antigenic site prediction.

MAbs B and C bind to an epitope between residues 288-302 in a region of the PHCS molecule which is highly flexible, as described by the main chain B-factor values shown in fig.7.3. However, their reactivity is greatly reduced when PHCS is held in a 'closed' form by bound

oxaloacetate (section 5.4). This effect may be caused solely by a change in the conformation of this epitope when oxaloacetate is bound. Alternatively, it may be that a reduction in the mobility of this epitope in the 'closed' form reduces antibody binding efficiency. A future study of the kinetics of binding of mAbs B and C to PHCS in the 'open' and 'closed' forms described, will enable further information to be gained regarding the mechanism of antibody-antigen interactions. A comparison of the binding affinities of mAbs B and C to the synthetic peptide 288-302, with their binding to peptides which differ by selected amino-acid substitutions, will further the understanding of these interactions.

A long-term project to compare the conformation of a synthetic peptide free in solution (from high field nuclear magnetic resonance spectroscopic data (Dyson et al, 1986) with its conformation when bound to a mAb (using data from NMR and X-ray crystallography) will show the extent of conformational change that an antibody confers upon binding to its epitope. If further X-ray crystallographic comparisons of the free 'open' and 'closed' forms of PHCS with those forms of the enzyme once bound by mAbs were performed it would also provide further information on this phenomenon.

CHAPTER NINEAPPENDIX

Page

9.1	Introduction	193
9.2	Immunofluorescence to show mAb binding to citrate synthase	193
9.3	Discussion	198

9.1 Introduction

In this appendix chapter I describe some preliminary work performed on the ability of mAbs to detect citrate synthase in fixed HEp-2 cells. This work was performed to show the feasibility of the approach, and some suggestions are made for further applications.

9.2 Immunofluorescence to show mAb binding to citrate synthase

HEp-2 cells a human epithelial cell line, were grown on multitest slides, and fixed according to the method described in section 3.11. Following incubation with mAbs or anti-serum, bound antibodies were detected using a fluorescent labelled second antibody, as described in section 3.12.

The reactivities of anti-PHCS antibodies with fixed HEp-2 cells is summarised in table 9.1. Only cytoplasmic staining was observed. Staining was assessed visually and assigned a value of - (negative), + (weak staining) or ++ (strong staining). Figures 9.1, 9.2 and 9.3 show examples of the cytoplasmic staining seen with normal mouse serum, mAb M and mAb D respectively.

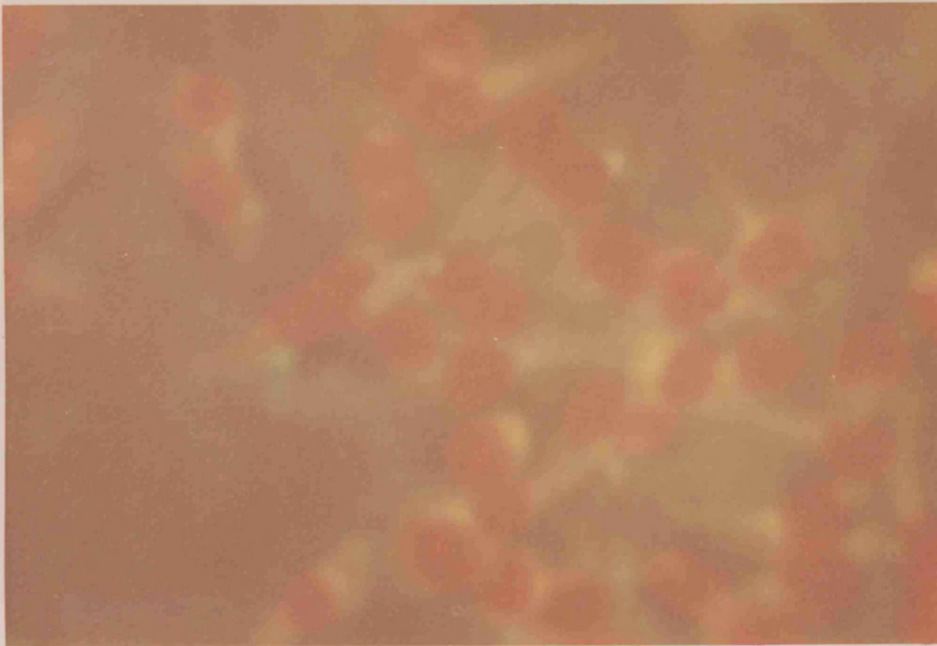
Table 9.1. Summary of the binding of mAbs and serum antibodies to fixed HEp-2 cells, shown by immunofluorescent staining

Antibody	Cytoplasmic staining
normal serum	-
anti-PHCS serum	+
mAb A	-
mAb B	-
mAb C	-
mAb D	++
mAb E	++
mAb F	+
mAb G	+
mAb H	+
mAb I	++
mAb L	-
mAb M	++

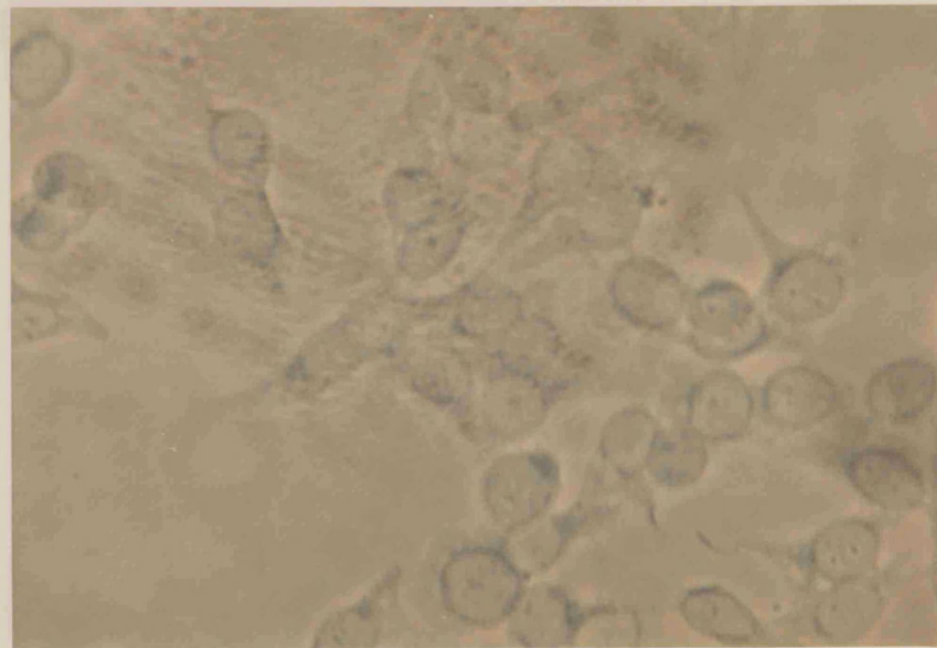
Assessment of cytoplasmic staining has been described in section 9.2.

Fig. 9.1 Reactivity of normal mouse serum with
fixed HEp-2 cells

a)



b)

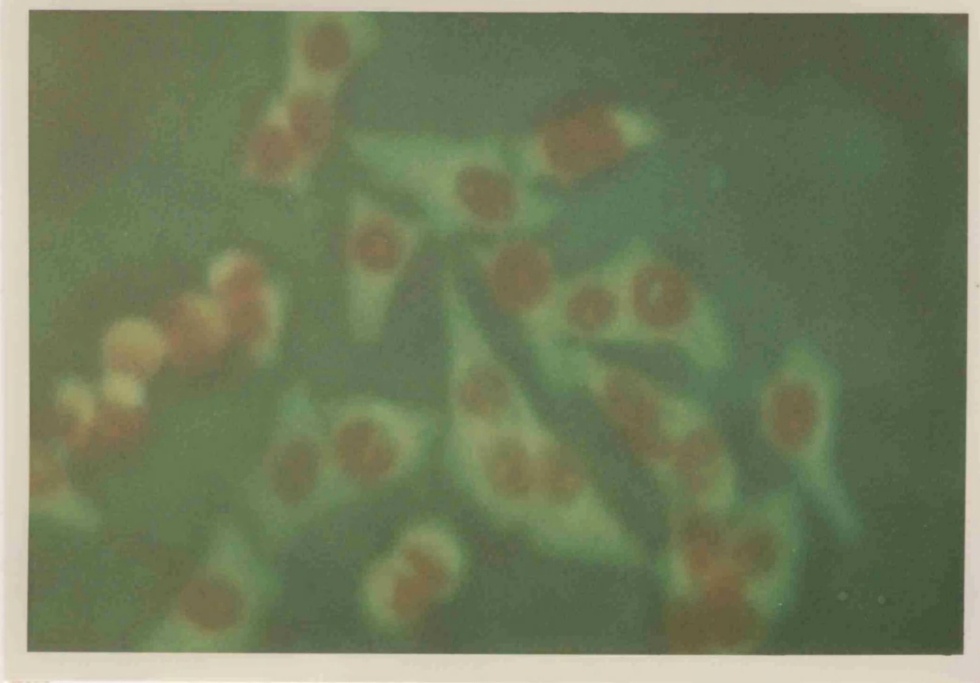


a) Immunofluorescent pattern shown under UV light.

b) Cells shown under light microscopy.

Fig 9.2 Reactivity of mAb M with fixed HEp-2 cells

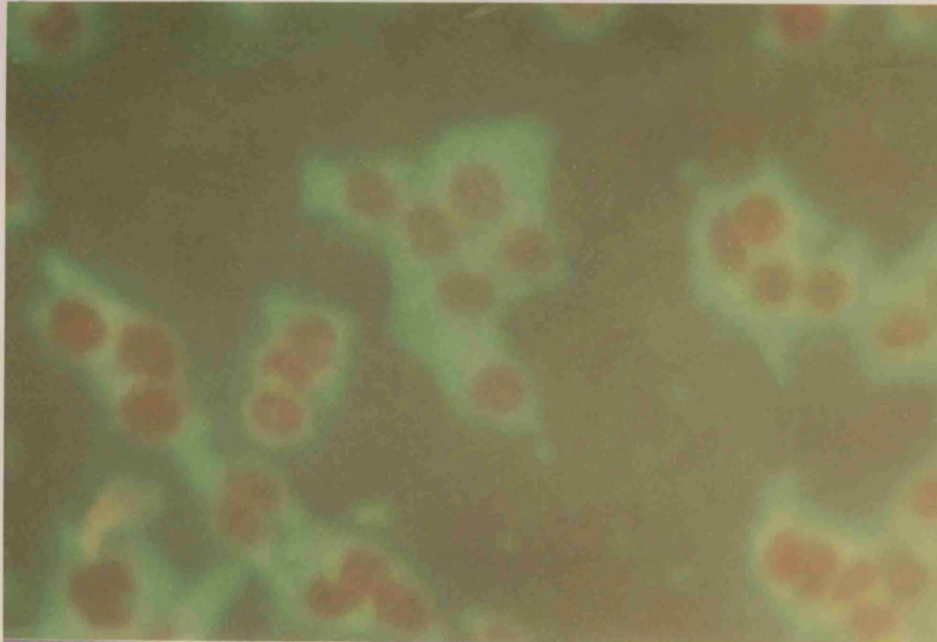
a)



a) Immunofluorescent pattern shown under UV light.

Fig. 9.3 Reactivity of mAb D with fixed HEp-2 cells

a)



antigen fixation may present the epitopes recognized by the four mAbs in a form which is no longer capable of binding to them.

This study provides preliminary information on the possible use of anti-citrate synthase mAbs in immunofluorescence experiments which may prove of value in further species cross-reactivity experiments.

Using electron-microscopy to study in finer detail the binding of mAbs to citrate synthase in fixed

a) Immunofluorescent pattern shown under UV light.

9.3 Discussion

This work shows that 7 of the 11 mAbs raised against PHCS bind to a cytoplasmic component, and can be visualised using immunofluorescence. Future work will have to prove that the binding of these mAbs is specific, to just citrate synthase. Proof of this may be achieved by running a cell lysate of HEp-2 cells on a gradient SDS-PAGE gel, and immunoblotting with mAbs. This will show if mAbs only bind to a lysate component which corresponds in molecular weight to citrate synthase.

The HEp-2 cell line is a human cell line. Four of the eleven mAbs do not bind to the antigen when assayed using immunofluorescence. This may reflect differences in the epitopes recognised by these mAbs between the PHCS and the human citrate synthase. Alternatively the method of antigen fixation may present the epitopes recognised by the four mAbs in a form which is no longer capable of binding to them.

This study provides preliminary information on the possible use of anti-citrate synthase mAbs in immunofluorescence experiments which may prove of value in further species cross-reactivity experiments.

Using electron-microscopy to study in finer detail the binding of mAbs to citrate synthase in fixed cells may provide further information on the orientation

of attachment of the enzyme to membranes. Further information may also be obtained into the association of other enzymes with citrate synthase, as discussed in section 2.2.

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